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state the following in connection with Australian Application No. 85114/91

1. We are the nominated person.
2. The nominated person is the assignee of the actual inventors.
3. Since filing the PCT application the representative of the nominated person has changed from The Secretary, United States Department of Commerce to The Secretary, United States Department of Agriculture.
4. The nominated person is the assignee of the applicants of the basic application listed in the declaration under Article 8 of the PCT.
5. The basic application is the first made in a convention country.

Dated: 26 May 1993

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To: The Commissioner of Patents

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Classification", replace "C12N 1:511" by "C12N  
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<p>(21) International Application Number: PCT/US91/06453</p> <p>(22) International Filing Date: 10 September 1991 (10.09.91)</p> <p>(30) Priority data: 579,896 10 September 1990 (10.09.90) US</p> <p>(71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE (US/US); Washington, DC 20231 (US). <b>AGRICULTURE</b></p> <p>(72) Inventors: THEOLOGIS, Athanasios ; 13434 Mandoli Drive, Los Altos Hills, CA 94022 (US). SATO, Takahide ; 15-36-8-204 Futciwarhigashi, Funabashi City, Chiba 280 (JP).</p>		<p>(74) Agents: OLIFF, James, A. et al.; Oliff &amp; Berridge, P.O. Box 19928, Alexandria, VA 22320 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p style="text-align: center; font-size: 2em;"><b>657276</b></p>
<p>(54) Title: RECOMBINANT ACC SYNTHASE</p> <p>(57) Abstract</p> <p>Isolation of the cDNA encoding the ACC synthase of zucchini using a novel isolation method permits the recovery of ACC synthase genes from a variety of higher plant sources. The ACC synthases are coded by multigene families, the members of which may be responsible for various plant development characteristics effected by ethylene. Recombinant production of ACC synthase and thereby <i>in vitro</i> production of ACC, ethylene and ethanol is also enabled by use of this gene. In addition, control of processes in plants which are mediated by ACC synthase can be effected using antisense technology or by the use of mutated ACC synthase genes.</p> <div style="text-align: center; margin-top: 100px;"></div>		

Field of the Invention

The invention relates to the plant enzyme ACC synthase which is essential for the production of ethylene in higher plants. More particularly, the invention concerns recombinant methods and materials for the production of this enzyme and their use in controlling plant development, and in particular, plant senescence.

Background Art

The enzyme ACC synthase is essential to the production of ethylene in higher plants. It is well known that ethylene is related to various events in plant growth and development including fruit ripening, seed germination, abscission, and leaf and flower senescence. Ethylene production is strictly regulated by the plant and is induced by a variety of external factors, including the application of auxins, wounding, anaerobic conditions, viral infection, elicitor treatment, chilling, drought, and ions such as cadmium and lithium ions. A review of ethylene production and effects in plants may be found, for example, in Abeles, F.B., "Ethylene in Plant Biology" (1983) Academic Press, New York.

It is also known that the synthesis of ethylene in higher plants includes a rate limiting step which is the conversion of S-adenosyl methionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC). This conversion is catalyzed by the enzyme ACC synthase (EC4.4.1.14). This enzyme has been partially purified from several sources by Nakajima, N., et al., Plant Cell Physiol (1986) 27:969-980; Mehta, A.M., et al., Proc Natl Acad Sci USA (1988) 85:8810-8814; Nakajima, N., et al., Plant Cell Physiol (1988) 29:989-990; Tsai, D.S., et al., Arch Biochem Biophys (1988) 264:632-640; Bleecker, A.B., et al., Proc Natl Acad Sci USA (1986) 83:7755-7759; Privale, L.S., et al., Arch Biochem Biophys (1987) 253:333-340; Sato, S., et al., Plant Physiol (1988) 88:109-114; Van Der Straeten, D., et al., Eur J Biochem (1989) 182:639-647.

As the level of ACC synthase controls the production

of ethylene, control of the level of this enzyme permits control of ethylene levels and thus regulation of the plant growth and development aspects that are controlled by ethylene. The availability of the ACC synthase gene, as provided by the invention herein, permits the construction of recombinant materials which permit such regulation. Furthermore, the availability of ACC synthase makes possible large-scale production of an ethylene precursor useful in industrial production of this chemical and its products, such as ethanol.

Subsequent to the present invention, Van Der Straeten, D., et al. reported the cloning and sequences of cDNAs encoding ACC synthase from tomato (Proc Natl Acad Sci USA (1990) 87:4859-4863). Although the cDNA, which corresponded to an open reading frame of approximately 55 kd, produced a 55 kd peptide in E. coli, it is not clear from the data provided that this protein represents ACC synthase.

#### Disclosure of the Invention

The invention provides recombinant materials and techniques which permit control of the level of ACC synthase in plants and portions thereof and also provides methods for large scale nonpetroleum-dependent production of ethylene. The recovery of a cDNA encoding the ACC synthase of zucchini provides access to recombinant materials corresponding to alternate ACC synthases from zucchini as well as the range of ACC synthases in higher plants. This permits the control of plant development and activity in a wide variety of plant materials of commercial interest.

Accordingly, in one aspect, the invention is directed to DNA in purified and isolated form consisting essentially of a DNA sequence encoding the enzyme ACC synthase of a higher plant. In other aspects, the invention is directed to expression systems effective in expressing the DNA encoding said ACC synthase, to recombinant hosts transformed with this expression system, and to methods of producing ACC synthase using these transformed hosts.

In other aspects, the invention is directed to methods to control ACC synthase production using the coding sequences for ACC synthase in an antisense construct or by replacing the ACC synthase gene by a mutated form thereof.

In another aspect, the invention is directed to a novel method to isolate an inducible cDNA without necessity for the purified protein which it encodes.

#### Brief Description of the Drawings

Figure 1A shows a restriction map of two clones encoding the zucchini ACC synthase enzyme; Figure 1B shows the nucleotide and deduced amino acid sequence of one of these clones.

Figure 2A-2C show elution patterns in chromatographic steps in the purification of ACC synthase from zucchini.

Figure 3 shows the elution pattern of the final step in the purification of ACC synthase from zucchini.

Figure 4 shows an SDS-polyacrylamide gel of the fractions of Figure 3.

Figure 5 shows a restriction map of genomic clones obtained by hybridization to the cDNA encoding zucchini ACC synthase. Figure 5A shows the alignment of the retrieved clones with the position of the coding sequences on the genome; Figure 5B shows a restriction map of the sequences on the genome; Figure 5C shows the functional portions of the two zucchini ACC synthase genes CP-ACC 1A and CP-ACC 1B.

Figure 6 shows the complete nucleotide sequence and deduced amino acid sequence of the genomic clone representing CP-ACC 1A. Both control regions and coding regions are shown.

Figure 7 shows the complete nucleotide sequence and deduced amino acid sequence of the genomic clone representing CP-ACC 1B. Both control regions and coding regions are shown.

Figure 8 shows the nucleotide and deduced amino acid sequence of a cDNA encoding the tomato ACC synthase.

Figure 9 shows a diagram and restriction map of

several clones of the cDNA encoding tomato ACC synthase.

Figure 10 shows a comparison of the amino acid sequence of the ACC synthase encoded by the zucchini genomic sequence CP-ACC 1A and the tomato genomic sequence ACC 2.

Figure 11 shows the pattern of genomic clones and functional diagrams thereof for the tomato genome containing coding and control sequences for LE-ACC 1A, LE-ACC 1B, and LE-ACC 3.

Figure 12 shows the pattern of genomic clones and the organization of the gene for LE-ACC 2.

Figure 13 shows the complete genomic and deduced amino acid sequence of LE-ACC 2, including the control sequences.

Figure 14 shows a comparison of the deduced amino acid sequence from the two genomic zucchini clones and the four genomic tomato clones for ACC synthase.

Figure 15 shows the junction region and a restriction map of a bacterial expression vector for the production of tomato ACC synthase in bacteria.

Figure 16 shows the production of ACC by bacterial cultures transformed with the vector of Figure 15 in the presence and absence of the inducer IPTG.

Figure 17 is a half tone photograph of a two-dimensional chromatographic gel of bacterial extracts wherein the bacterial culture is transformed with an expression vector for tomato ACC synthase having the coding sequence in the correct and incorrect orientations.

Figure 18 shows the construction of an expression vector for the tomato ACC-synthase gene oriented in the antisense direction.

Figures 19 and 20 show the ethylene production by tomato plants regenerated from tomato cotyledons transformed with the vector of Figure 18 as a function of days from pollination.

#### Modes of Carrying Out the Invention

The presence of ACC synthase as the controlling factor in ethylene production appears to be universal in

higher plants. As a result of the recovery of cDNA according to the invention, as described in Sato, T. and Theologis, A., Proc Natl Acad Sci USA (1989) 86:6621-6625, mailed to subscribers September 11, 1989, and incorporated herein by reference, the presence of a gene family encoding a number of homologous, but different, ACC synthases in zucchini has been shown. The various ACC synthases control ethylene production at various locations in the differentiated plant, thus permitting separate control of, for example, fruit ripening and seed germination. In addition, the availability of this cDNA and the resulting genomic DNAs has permitted the recovery of DNAs encoding ACC synthases in other plants, including, for example, tomato. While the various ACC synthases are generally active in a variety of plant tissues, the DNAs are not completely homologous, and therefore the use of the genetic materials for control of synthesis, for example, using an antisense strategy, does not translate cross-species.

#### Definitions

As used herein, "recombinant" refers to a nucleic acid sequence which has been obtained by manipulation of genetic material using restriction enzymes, ligases, and similar recombinant techniques as described by, for example, Maniatis et al. "Recombinant", as used in the present application, does not refer to naturally-occurring genetic recombinations.

As defined herein, "ACC synthase" includes all enzymes which are capable of catalyzing the conversion of AdoMet to ACC and methyl thioadenosine (MTA). The amino acid sequence of the synthase may or may not be identical with the amino acid sequence which occurs natively in higher plants. An example of such native sequence is shown in Figure 1 which occurs in the zucchini fruit (Cucurbita pepo). Naturally occurring allelic variants undoubtedly occur as well. Similar proteins are present in a wide variety of higher plants. In addition, artificially induced mutations are also included so long as they

do not destroy activity. In general, conservative amino acid substitutions can be made for most of the amino acids in the primary structure as shown without affecting destruction of activity. Thus, the definition of ACC synthase used herein includes these variants which are derived by direct or indirect manipulation of the disclosed sequences.

It is also understood that the primary structure may be altered by post-translational processing or by subsequent chemical manipulation to result in a derivatized protein which contains, for example, glycosylation substituents, oxidized forms of, for example, cysteine or proline, conjugation to additional moieties, such as carriers, solid supports, and the like. These alterations do not remove the protein from the definition of ACC synthase so long as its capacity to convert AdoMet to ACC and MTA is maintained.

Thus, the identity of an enzyme as "ACC synthase" can be confirmed by its ability to effect the production of ethylene in an assay performed as follows: the enzyme to be tested is incubated with 200  $\mu$ M AdoMet, 10  $\mu$ M pyridoxal phosphate, 40  $\mu$ g BSA in 200 mM Hepes buffer, pH 8.5 in a total volume of 600  $\mu$ l at 30°C for 30 minutes, and the amount of ACC formed is assayed by conversion to ethylene using hypochlorite as described, for example, by Lisada, C.C., et al., Anal Biochem (1979) 100:140-145. While alternative forms of assessment of ACC synthase can be devised, and variations on the above protocol are certainly permissible, the foregoing provides a definite criterion for the presence of ACC synthase activity and classification of a test protein as ACC synthase.

The amino acid sequences for several ACC synthases in tomato and zucchini are shown in Figures 1, 8 and 14. Preferred forms of the ACC synthases of the invention include those thus illustrated herein, and those derivable therefrom by systematic mutation of the genes. Such systematic mutation may be desirable to enhance the ACC synthase properties of the enzyme, to enhance the



characteristics of the enzyme which are ancillary to its activity, such as stability, or shelf life, or may be desirable to provide inactive forms useful in the control of ACC activity in vivo.

5 As described above, "ACC synthase" refers to a protein having the activity assessed by the assay set forth above; a "mutated ACC synthase" refers to a protein which does not necessarily have this activity, but which is derived by mutation of a DNA encoding an ACC synthase.  
10 By "derived from mutation" is meant both direct physical derivation from a DNA encoding the starting material ACC synthase using, for example, site specific mutagenesis or indirect derivation by syntheses of DNA having a sequence related to, but deliberately different from, that of the  
15 ACC synthase. As means for constructing oligonucleotide of the required length are available, such DNAs can be constructed wholly or partially from their individual constituent nucleotides.

As used herein, "higher plant" refers to those plants  
20 whose development and activity are controlled by ethylene. These includes all common agricultural plants and various flowering species.

#### Initial Isolation of the ACC Synthase cDNA

25 The ACC synthase cDNA was isolated initially from Cucurbita pepo (zucchini) using a novel method which is applicable to inducible proteins. The method does not require pure protein in order to design probes or to prepare monoclonal antibodies; the method relies on the production of a cDNA expression library from induced  
30 tissue and identification of positive clones using an antibody preparation which has been purified by taking advantage of the inducible nature of the protein. Thus, in general, the method comprises the steps of preparing partially purified inducible protein of interest from the  
35 cells or tissue which have been induced for this production and preparing a composition of antibodies to these purified proteins. The composition of antibodies is prepared in a conventional manner by immunization of a

suitable mammal with a protocol designed to enhance the production of antiserum.

The first composition of antibodies, which will also contain antibodies to the protein contaminants in the preparation, is then purified to obtain a second antibody composition enriched in the antibodies immunoreactive for the desired protein. This enrichment is effected by reacting the first prepared composition with a protein extract from uninduced tissue. This will selectively remove those antibodies immunoreactive with background contaminants. This purified preparation of antibodies is then used to screen a cDNA expression library which has been prepared from tissue expressing the gene encoding the inducible protein. As the purified antibody preparation is immunospecific for this protein, identification of the positive clones is simplified. The application of this method to the recovery of cDNA encoding ACC synthase is described in detail in Example 1 herein. However, a similar method can be used to obtain the cDNA for any inducible protein, even without isolation and purification of the desired target protein.

#### Extension of the ACC Synthase Family

The availability of the cDNA encoding zucchini ACC synthase makes accessible both the multigene family which provides the variety of ACC synthases found in the same plant host-i.e., zucchini, as well as other cDNAs encoding ACC synthase from other species of higher plants and their corresponding multiple gene families. The cDNAs or portions thereof are used as probes to hybridize to the additional genomic or cDNA sequences by hybridization under standard conditions. Typical standard conditions of stringency include those set forth, for example, in Example 4. These recovered sequences can also be engineered to effect the expression of ACC synthase, to make modification which result in ACC synthase mutants, or "mutated ACC synthase" and to construct antisense vectors to control the production of indigenous ACC synthase.

Recombinant Production of ACC Synthase

The availability of the ACC synthase gene permits its production in a variety of recombinant systems. Recombinant production of this enzyme in single cellular systems, including procaryotic and eucaryotic systems, provides the tools for the recombinant production of ethylene and ethanol, the products of the ACC synthesized. Large scale production of these chemicals can be effected by utilizing suitable large scale recombinant production of the ACC synthase enzyme to effect the endogenous production of ACC followed by chemical conversion of the ACC to ethylene and/or ethanol. In order to make such production economically attractive, large scale production, such as in large algae cultures, is preferred. The ACC synthase can also be produced in transgenic plants both in enhanced amounts and in an antisense mode, as further set forth below, to control the aspects of plant development which are ethylene sensitive, and in particular, to delay plant senescence.

Accordingly, a variety of expression systems and hosts can be used for the production of this enzyme. A variety of procaryotic hosts and appropriate vectors is known in the art; most commonly used are E. coli or other bacterial hosts such as B. subtilis or Pseudomonas and typical bacterial promoters include the trp, lac, tac, and  $\beta$ -lactamase promoters. A readily controllable, inducible promoter, the  $\lambda$ -phage promoter can also be used. A large number of control systems suitable for procaryote expression is known in the art.

Similarly, a large number of recombinant systems have been developed for expression in eucaryotic hosts, including yeast, insect cells, mammalian cells, and plant cells. These systems are well characterized, and require the ligation of the coding sequence under the control of a suitable transcription initiating system (promoter) and, if desired, termination sequences and enhancers. Especially useful in connection with the ACC synthase genes of the present invention are expression systems

which are operable in plants. These include systems which are under control of a tissue-specific promoter, as well as those which involve promoters that are operable in all plant tissues.

5           Transcription initiation regions, for example, include the various opine initiation regions, such as octopine, mannopine, nopaline and the like. Plant viral promoters can also be used, such as the cauliflower mosaic virus 35S promoter. In addition, plant promoters such as  
10           ribulose-1,3-diphosphate carboxylase, fruit-specific promoters, heat shock promoters, seed-specific promoters, etc. can also be used.

          The cauliflower mosaic virus (CaMV) 35S promoter has been shown to be highly active in many plant organs and  
15           during many stages of development when integrated into the genome of transgenic plants including tobacco and petunia, and has been shown to confer expression in protoplasts of both dicots and monocots.

          The CaMV 35S promoter has been demonstrated to be  
20           active in at least the following monocot and dicot plants with edible parts: blackberry, Rubus; blackberry/raspberry hybrid, Rubus, and red raspberry; carrot, Daucus carota; maize; potato, Solanum tuberosum; rice, Oryza sativa; strawberry, Fragaria x ananassa; and tomato, Lycopersicon esculentum.  
25           

          The nopaline synthase (Nos) promoter has been shown to be active in at least the following monocot and dicot plants with edible parts: apple, Malus pumila; cauliflower, Brassica oleracea; celery, Apium graveolens;  
30           cucumber, Cucumis sativus; eggplant, Solanum melongena; lettuce, Lactuca sativa; potato, Solanum tuberosum; rye, Secale cereale; strawberry, Fragaria x ananassa; tomato, Lycopersicon esculentum; and walnut, Juglans regia.

          Organ-specific promoters are also well known. For  
35           example, the E8 promoter is only transcriptionally activated during tomato fruit ripening, and can be used to target gene expression in ripening tomato fruit (Deikman and Fischer, EMBO J (1988) 7:3315; Giovannoni et al., The

Plant Cell (1989) 1:53). The activity of the E8 promoter is not limited to tomato fruit, but is thought to be compatible with any system wherein ethylene activates biological processes.

5 Other organ-specific promoters appropriate for a desired target organ can be isolated using known procedures. These control sequences are generally associated with genes uniquely expressed in the desired organ. In a  
10 typical higher plant, each organ has thousands of mRNAs that are absent from other organ systems (reviewed in Goldberg, Phil, Trans R Soc London (1986) B314:343).

These mRNAs first isolated to obtain suitable probes for retrieval of the appropriate genomic sequence which retains the presence of the natively associated control  
15 sequences. An example of the use of techniques to obtain the cDNA associated with mRNA specific to avocado fruit is found in Christoffersen et al., Plant Molecular Biology (1984) 3:385. Briefly, mRNA was isolated from ripening  
20 avocado fruit and used to make a cDNA library. Clones in the library were identified that hybridized with labeled RNA isolated from ripening avocado fruit, but that did not hybridize with labeled RNAs isolated from unripe avocado  
25 fruit. Many of these clones represent mRNAs encoded by genes that are transcriptionally activated at the onset of avocado fruit ripening.

A somewhat more sophisticated procedure was described in Molecular Biology of the Cell, Second Edition (1989) pages 261-262, edited by Alberts et al., Garland  
30 Publishing Incorporated, New York. In this procedure, mRNAs enriched for organ-specific nucleic acid sequences were used to construct the cDNA library. This method was also applied to tomato by Lincoln et al., Proc Natl Acad Sci (1987) 84:2793, and resulted in the production of an  
E8 cDNA clone.

35 The gene that encodes the organ-specific mRNA is then isolated by constructing a library of DNA genomic sequences from the plant. The genome library is screened with the organ-specific DNA clone, and the sequence is

determined. The promoter is then isolated. These procedures are now considered to be routine and are described in detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Either a constitutive promoter or a desired organ-specific promoter is then ligated to the gene encoding ACC synthase or a mutated form thereof using standard techniques now common in the art. The expression system may be further optimized by employing supplemental elements such as transcription terminators and/or enhancer elements.

Thus, for expression in plants, the recombinant expression cassette will contain in addition to the ACC synthase-encoding sequence, a plant promoter region, a transcription initiation site (if the coding sequence to be transcribed lacks one), and a transcription termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette are typically included to allow for easy insertion into a pre-existing vector.

Sequences controlling eucaryotic gene expression have been extensively studied. Promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs (bp) upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. By convention, the start site is called +1. Sequences extending in the 5' (upstream) direction are given negative numbers and sequences extending in the 3' (downstream) direction are given positive numbers.

In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G(or T)NG (Messing, J. et al., in Genetic Engineering in Plants, Kosage, Meredith and Hollaender, eds. (1983) pp. 221-227). Other sequences conferring tissue specificity, response to environmental signals, or maximum efficiency of transcription may also be found in the

promoter region. Such sequences are often found within 400 bp of transcription initiation site, but may extend as far as 2000 bp or more.

5 In the construction of heterologous promoter/structural gene combinations, the promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be  
10 accommodated without loss of promoter function.

As stated above, any of a number of promoters which direct transcription in plant cells is suitable. The promoter can be either constitutive or inducible. Promoters of bacterial origin include the octopine synthase promoter, the nopaline synthase promoter and other  
15 promoters derived from native Ti plasmids (Herrera-Estrella et al., Nature (1983) 303:209-213). Viral promoters include the 35S and 19S RNA promoters of cauliflower mosaic virus (O'Dell et al., Nature (1985) 313:810-  
20 812). Plant promoters include the ribulose-1,3-diphosphate carboxylase small subunit promoter and the phaseolin promoter. The promoter sequence from the E8 gene and other genes in which expression is induced by ethylene may also be used. The isolation and sequence of  
25 the E8 promoter is described in detail in Deikman and Fischer, EMBO J (1988) 7:3315-3320 which is incorporated herein by reference.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination  
30 region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

If the mRNA encoded by the structural gene is to be  
35 efficiently processed, DNA sequences which direct polyadenylation of the RNA are also commonly added to the vector construct (Albert and Kawasaki, Mol and Appl Genet, (1982) 1:419-434). Polyadenylation is of importance for

expression of the ACC synthase-encoding RNA in plant cells. Polyadenylation sequences include, but are not limited to the Agrobacterium octopine synthase signal (Gielen et al., EMBO J, (1984) 3:835:-846) or the nopaline synthase signal (Depicker et al., Mol and Appl Genet (1982) 1:561-573).

The resulting expression system or cassette is ligated into or otherwise constructed to be included in a recombinant vector which is appropriate for higher plant transformation. The vector will also typically contain a selectable marker gene by which transformed plant cells can be identified in culture. Usually, the marker gene will encode antibiotic resistance. These markers include resistance to G418, hygromycin, bleomycin, kanamycin, and gentamicin. After transforming the plant cells, those cells having the vector will be identified by their ability to grow on a medium containing the particular antibiotic. Replication sequences, of bacterial or viral origin, are generally also included to allow the vector to be cloned in a bacterial or phage host, preferably a broad host range procaryotic origin of replication is included. A selectable marker for bacteria should also be included to allow selection of bacterial cells bearing the desired construct. Suitable procaryotic selectable markers also include resistance to antibiotics such as kanamycin or tetracycline.

Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art. For instance, in the case of Agrobacterium transformation, T-DNA sequences will also be included for subsequent transfer to plant chromosomes.

In addition, vectors can also be constructed that contain in-frame ligations between the sequence encoding the ACC synthase protein and sequences encoding other molecules of interest resulting in fusion proteins, by techniques well known in the art.

When an appropriate vector is obtained, transgenic plants are prepared which contain the desired expression



system. A number of techniques are available for transformation of plants or plant cells. All types of plants are appropriate subjects for "direct" transformation; in general, only dicots can be transformed using *Agrobacterium*-mediated infection.

In one form of direct transformation, the vector is microinjected directly into plant cells by use of micro-pipettes to mechanically transfer the recombinant DNA (Crossway, Mol Gen Genetics (1985) 202:179-185). In another form, the genetic material is transferred into the plant cell using polyethylene glycol (Krens, et al., Nature (1982) 296:72-74), or high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface, is used (Klein, et al., Nature (1987) 327:70-73). In still another method protoplasts are fused with other entities which contain the DNA whose introduction is desired. These entities are minicells, cells, lysosomes or other fusible lipid-surfaced bodies (Fraley, et al., Proc Natl Acad Sci USA (1982) 79:1859-1863).

DNA may also be introduced into the plant cells by electroporation (Fromm et al., Proc Natl Acad Sci USA (1985) 82:5824). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide and regenerate.

For transformation mediated by bacterial infection, a plant cell is infected with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the DNA to be introduced. *Agrobacterium* is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by

the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of A. tumefaciens or the Ri plasmid of A. rhizogenes. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome (Schell, J., Science (1987) 237:1176-1183). Ti and Ri plasmids contain two regions essential for the production of transformed cells. One of these, named transferred DNA (T-DNA), is transferred to plant nuclei and induces tumor or root formation. The other, termed the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. The T-DNA will be transferred into a plant cell even if the vir region is on a different plasmid (Hoekema, et al., Nature (1983) 303:179-189). The transferred DNA region can be increased in size by the insertion of heterologous DNA without its ability to be transferred being affected. Thus a modified Ti or Ri plasmid, in which the disease-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell.

Construction of recombinant Ti and Ri plasmids in general follows method typically used with the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to "shuttle vectors," (Ruvkun and Ausubel, Nature (1981) 298:85-88), promoters (Lawton et al., Plant Mol Biol (1987) 9:315-324) and structural genes for antibiotic resistance as a selection factor (Fraley et al., Proc Natl Acad Sci (1983) 80:4803-4807).

There are two classes of recombinant Ti and Ri

plasmid vector system now in use. In one class, called "cointegrate," the shuttle vector containing the gene of interest is inserted by genetic recombination into a non-oncogenic Ti plasmid that contains both the cis-acting and trans-acting elements required for plant transformation as, for example, in the pMLJ1 shuttle vector of DeBlock et al., EMBO J (1984) 3:1681-1689 and the non-oncogenic Ti plasmid pGV2850 described by Zambryski et al., EMBO J (1983) 2:2143-2150. In the second class or "binary" system, the gene of interest is inserted into a shuttle vector containing the cis-acting elements required for plant transformation. The other necessary functions are provided in trans by the non-oncogenic Ti plasmid as exemplified by the pBIN19 shuttle vector described by Bevan, Nucleic Acids Research (1984) 12:8711-8721 and the non-oncogenic Ti plasmid PAL4404 described by Hoekma, et al., Nature (1983) 303:179-180. Some of these vectors are commercially available.

There are two common ways to transform plant cells with Agrobacterium: co-cultivation of Agrobacterium with cultured isolated protoplasts, or transformation of intact cells or tissues with Agrobacterium. The first requires an established culture system that allows for culturing protoplasts and subsequent plant regeneration from cultured protoplasts. The second method requires (a) that the intact plant tissues, such as cotyledons, can be transformed by Agrobacterium and (b) that the transformed cells or tissues can be induced to regenerate into whole plants.

Most dicot species can be transformed by Agrobacterium as well as species which are a natural plant host for Agrobacterium are transformable in vitro. Monocotyledonous plants, and in particular, cereals, are not natural hosts to Agrobacterium. Attempts to transform them using Agrobacterium have been unsuccessful until recently (Hooykas-Van Slogteren et al., Nature (1984) 311:763-764). However, there is growing evidence now that certain monocots can be transformed by Agrobacterium.

Using novel experimental approaches cereal species such as rye (de la Pena et al., Nature (1987) 325:274-276), maize (Rhodes et al., Science (1988) 240:204-207), and rice (Shimamoto et al., Nature (1989) 338:274-276) may now be transformed.

Identification of transformed cells or plants is generally accomplished by including a selectable marker in the transforming vector, or by obtaining evidence of successful bacterial infection.

Plant cells which have been transformed can also be regenerated using known techniques.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. II, 1986). It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugar-cane, sugar beet, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, somatic embryo formation can be induced in the callus tissue. These somatic embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and plant hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

A large number of plants have been shown capable of regeneration from transformed individual cells to obtain

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transgenic whole plants. For example, regeneration has been shown for dicots as follows: apple, Malus pumila; blackberry, Rubus, Blackberry/raspberry hybrid, Rubus, red raspberry, Rubus; carrot, Daucus carota; cauliflower, Brassica oleracea; celery, Apium graveolens; cucumber, Cucumis sativus; eggplant, solanum melongena; lettuce, Lactuca sativa; potato, Solanum tuberosum; rape, Brassica napus; soybean (wild), Glycine canescens; strawberry, Fragaria x ananassa; tomato, Lycopersicon esculentum; walnut, Juglans regia; melon, Cucumis melo; grape, Vitis vinifera; mango, Mangifera indica;

and for the following monocots: rice, Oryza sativa; rye, Secale cereale; and maize.

In addition, regeneration of whole plants from cells (not necessarily transformed) has been observed in: apricot, Prunus armeniaca; asparagus, Asparagus officinalis; banana, hybrid Musa; bean, Phaseolus vulgaris; cherry, hybrid Prunus; grape, Vitis vinifera; mango, Mangifera indica; melon, Cucumis melo; ochra, Abelmoschus esculentus; onion, hybrid Allium; orange, Citrus sinensis; papaya, Carrica papaya; peach, Prunus persica and plum, Prunus domestica; pear, Pyrus communis; pineapple, Ananas comosus; watermelon, Citrullus vulgaris; and wheat, Triticum aestivum.

The regenerated plants are transferred to standard soil conditions and cultivated in a conventional manner.

After the expression cassette is stably incorporated into regenerated transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

The plants are grown and harvested using conventional procedures.

#### Antisense Expression

When the ACC synthase coding sequence is placed in correct orientation in the expression systems described above, the ACC synthase protein is produced. However, when placed in the opposite orientation, the expression vector

has an antisense effect which can interfere with the indigenous production of this enzyme. Antisense technology can work at a variety of levels including hybridization to a messenger RNA encoding the ACC synthase, hybridization to single-stranded intermediates in the production of this mRNA, or triplex formation with the DNA duplex which contains the ACC synthase genes. All of these modalities can be employed in effecting antisense control of ACC synthase production.

As shown in Example 8 below, ripening of tomato fruit can be controlled and inhibited by suitable antisense expression of the ACC synthase coding sequence supplied in a vector under the control of the cauliflower 35S promoter. Other properties which are controlled by ethylene can also be influenced by appropriate choice of control systems and/or the particular ACC synthase encoded.

It is further shown below that the active form of ACC synthase in higher plants is a dimer. By supplying a mutated form of ACC synthase monomer, a decoy can be produced to obtain inactive monomer and thereby regulate the levels of ACC synthase in the plant. An additional embodiment of the invention involves the mutated ACC synthase and expression systems therefor.

The following examples are intended to illustrate but not to limit the invention.

#### Example 1

##### Recovery of Zucchini ACC Synthase cDNA

A cDNA encoding ACC synthase in zucchini fruit was recovered as follows:

Slices 1 mm thick were prepared from zucchini fruits of the species Cucurbita pepo. To induce production of ACC synthase, slices were incubated for 18-24 hours in induction medium (50  $\mu$ M potassium phosphate buffer, pH 6.8; 0.5 mM indole acetic acid (IAA); 0.1 mM benzyl adenine (BA); 50 mM LiCl; 0.6 mM aminooxyacetic acid (AOA); and 50  $\mu$ g/ml chloramphenicol. (Uninduced tissue was prepared in a similar manner in 50 mM phosphate buffer, pH 6.8.)

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Poly(A<sup>+</sup>) RNA (mRNA) was isolated from 18-hr tissue prepared as described above, and in vitro translated in a wheat germ lysate as described by Theologis, A., et al., J Mol Biol (1985) 183:53-68, in the presence of labeled methionine (greater than 1,000 Ci/ $\mu$ mol) to verify the presence of ACC synthase encoding mRNA. A cDNA library was prepared in  $\lambda$ gt11 as described by Huynh, T.V., et al., in "DNA Cloning Techniques," Glover, E., ed. (1985) IRL Press, Oxford, 1:49-88. The insert sizes were 200-500 bp. The library was screened with purified ACC synthase anti-serum prepared as follows:

The antisera were prepared to 1500-fold purified ACC synthase preparations. Purified ACC synthase can be prepared from tissue homogenates sequentially bound to and eluted from Butyl Toyopearl (Toyo Soda Tokyo), SP-Sephadex, and QAE-Sephadex. (Higher purification can be obtained by subsequent chromatography sequentially through columns containing Butyl Toyopearl, Sephacryl S-300, Bio Gel-Ht, and finally FPLC mono-Q. The application of all of the following steps results in approximately a 6000-fold purification.) The antibodies are prepared in New Zealand white rabbits by immunization protocols involving four immunizations at three-week intervals with 5000 nmol of ACC synthase activity/hr (specific activity 1500 nmol of ACC/hr/mg protein obtained from the Bio Gel-HT column). Crude antiserum (2 ml) was purified by incubation with 10 ml Sepharose 4B coupled with soluble proteins from intact noninduced Cucurbita fruit. This step removed antibodies immunoreactive with protein other than ACC synthase.)

Sixty-six immunoreactive clones were isolated by screening  $1.4 \times 10^5$   $\lambda$ gt11 recombinant clones with the purified antiserum. Upon rescreening, only 30 were, in fact, positive. Southern analysis showed that 19 clones represented the ACC synthase mRNA. One selected clone, pACC1, has an open reading frame encoding a 55.8 kd polypeptide. Another intensely immunoreactive clone, pACC7, was much shorter. Figure 1A shows a restriction map of pACC1 and pACC7; Figure 1B shows the complete

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nucleotide sequence and deduced amino acid sequence for these clones.

As shown in Figure 1B, pACC7 is identical to a portion of the sequence of pACC1. The open reading frame encodes a protein of 493 amino acids, corresponding to a 55.779 kd polypeptide.

The positive clones from the  $\lambda$ gt11 library could also be used to prepare further purified antiserum for immunoblotting as follows:

The positive clones from the expression library was plated on E. coli strain Y1090 to obtain  $10^5$  plaque-forming units per 90-mm plate. Dry nitrocellulose filters presoaked in 10 mM isopropyl  $\beta$ -D-thiogalacto-pyranoside (IPTG) were laid on the lawn after incubation for two hours at 42°C and then incubated for an additional four hours at 37°C.

The filters were then soaked for 30 minutes in TBST (50 mM Tris HCl, pH 8.0; 0.14M NaCl; 0.05% Tween 20); 2% milk protein and then tested for ACC synthase expression by treating with 5 ml of diluted (1:500) purified ACC synthase antiserum (see below) per filter for two hours.

After washing five times at 10 minutes each with TBST, bound antibody was eluted by shaking for three minutes at room temperature with 0.2M glycine hydrochloride buffer, pH 2.3, containing 1% milk protein. The antibody solution was neutralized and used for immunoblotting.

#### Example 2

##### Purification of Native ACC Synthase From Cucurbita

ACC synthase was purified 6000-fold from induced Cucurbita homogenates according to a multistep protocol as shown below. Various buffers used in the purification are as follows:

Buffer A: Tris-HCl 100 mM, pH 8.0, EDTA 20 mM, pyridoxal phosphate 10  $\mu$ M, PMSF 0.5 mM,  $\beta$ -mercaptoethanol 20 mM; Buffer B: Tris-HCl 20 mM, pH 8.0, EDTA 10 mM, pyridoxal phosphate 10  $\mu$ M, DTT 0.5 mM; Buffer C: Na-acetate 20 mM, pH 6.0, pyridoxal phosphate 10  $\mu$ M, EDTA 10



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mM, DTT 0.5 mM; Buffer D: K-phosphate 10 mM, pH 8.0, pyridoxal phosphate 10  $\mu$ M, EDTA 1mM, DTT 0.5 mM; Buffer E: Tris-HCl 20mM, pH 8.0, pyridoxal phosphate 5  $\mu$ M, EDTA 1 mM, DTT 0.5 mM; Buffer F: Hepes-KOH 500 mM, pH 8.5, pyridoxal phosphate 40  $\mu$ M, BSA 400  $\mu$ g/ml.

All operations were performed at 4°C. Chromatographic elutions were assayed for ACC synthase activity and by absorption at 280 nm.

Ten kg of Cucurbita slices incubated for 24 hr in induction medium were chilled with liquid N<sub>2</sub> and homogenized in batches of 2 kg with 2 liters of buffer A plus 200g of polyvinylpyrrolidone in a one gallon Waring blender for 1 min at medium speed. The homogenate was centrifuged at 17,000 x g for 30 min. The supernatant was filtered through one layer of microcloth and one layer of nylon cloth (30  $\mu$ m mesh).

#### Butyl Toyopearl Fractionation

Solid ammonium sulfate was added slowly to the stirred supernatant above to achieve 40% saturation. The supernatant solution was stirred for 15 min and 300 ml of packed Butyl Toyopearl 650 M hydrophobic affinity matrix, previously equilibrated with buffer B saturated to 40% with ammonium sulfate, were added. The suspension was occasionally stirred for an additional 30 min. The matrix was recovered by filtration through one layer of nitex nylon cloth (30  $\mu$ m mesh) and the solution was squeezed out by hand. Subsequently, the matrix was placed in a vacuum filter with two sheets of Whatman filter paper #1 and washed with 500 ml of buffer B containing 40% ammonium sulfate. The adsorbed proteins were eluted from the matrix by washing (twice) with 750 ml of buffer B, batch-wise. The combined eluates were dialyzed three times against 10 liters of buffer B for 36 hr.

#### SP-Sephadex Fractionation

The dialyzed fraction above was clarified by centrifugation at 17,000 x g for 30 min. The volume was adjusted to 4 liters with buffer B and the pH was brought to pH 6.0 with 5% acetic acid. Two liters of packed

SP-Sephadex C-50 equilibrated with buffer C were added and the suspension was stirred for 60 min. The matrix was recovered by filtration through two sheets of Whatman filter paper #1 and washed with 2 liters of buffer C. The adsorbed proteins were eluted twice with 1 liter of buffer B containing 1M KCl, batchwise. The eluant was recovered by suction through #1 Whatman filter paper and solid ammonium sulfate was added to achieve 40% saturation. Subsequently 100 ml of Butyl Toyopearl-packed matrix equilibrated with buffer B/40% ammonium sulfate was added to the eluate. The suspension was stirred for 30 min and the matrix was collected by filtration through a layer of Nitex nylon cloth (30  $\mu$ m mesh). The matrix was resuspended in a small volume of buffer B/40% ammonium sulfate and poured in a column (2.5 x 20 cm). The adsorbed proteins were eluted with buffer B, and the flow rate of the column was under gravity. Fractions with high  $A_{280}$  were pooled and dialyzed overnight against 4 liters buffer B with three buffer changes during the course of dialysis.

#### QAE-Sephadex Fractionation

Four hundred ml of packed QAE-Sephadex equilibrated with buffer B were added to the dialyzate from the SP-Sephadex fractionation and the suspension was stirred gently for 60 min. The matrix was recovered by filtration through a layer of miracloth in a filtration apparatus and washed with 500 ml of buffer B to remove unadsorbed proteins. The matrix was resuspended in a small volume of buffer B and poured into a column (4 x 30 cm). The proteins were eluted with buffer B containing 0.2M KCl.

#### Butyl Toyopearl Chromatography

Solid ammonium sulfate was added to the eluate (~100 ml) to achieve 40% saturation and the solution was kept at 4°C for at least 4 hr. The suspension was centrifuged at 30,000 x g for 30 min and the supernatant was applied on a Butyl Toyoperl column (1.5 x 14 cm) equilibrated with buffer B/40% ammonium sulfate. After all the protein solution was passed through the column, it was eluted with a 400 ml linear gradient: 40 to 0% ammonium sulfate in

buffer B with a flow rate of 1 ml/min.

Figure 2A shows the elution pattern. Solid ammonium sulfate was added to enzymatically active fractions to achieve 80% saturation and the solution was incubated at 4°C for at least 4 hr. The precipitate was collected by centrifugation at 30,000 x g for 30 min at 4°C and dissolved in 3 ml of buffer D.

#### Sephacryl S-300 Chromatography

The resulting protein solution from above was applied to a column (2.5 x 100 cm) of Sephacryl S-300 equilibrated with buffer D. The column was eluted with buffer D at a flow rate of 0.5 ml/min. Figure 2B shows the elution pattern.

#### Bio Gel-HT Chromatography

Active fractions from the Sephacryl step were combined and applied on a Bio Gel-HA column (0.75 x 14 cm) equilibrated with buffer D. The column was washed with buffer D until  $A_{280} = 0$  and it was then eluted with a 200 ml linear gradient: 10-100 mM potassium phosphate in buffer D with a flow rate of 0.1 ml/min. Figure 2C shows the elution pattern. The active fractions were collected and concentrated with a Centricon 30 filtration apparatus, concomitantly the buffer of the concentrated protein solution was changed to buffer E.

#### FPLC Mono-Q Chromatography

The concentrated active fractions (~0.5 ml) from the Bio Gel-HT column were applied to a mono-Q H5/5 column. The column was washed with buffer E containing 0.1M KCl until  $A_{280} = 0$ . The column then was eluted with a 15 ml linear gradient: 0.1 to 0.4M KCl in buffer E. The flow rate of the gradient was 0.5 ml/min. Figure 3 shows the elution pattern.

Table 1 shows the overall purification sequence and the increase in specific activity with each successive step. The overall process results in a 6000-fold purification with a recovery of 7.5%. The enzyme has a specific activity of 35,590 nmol ACC produced /hr/mg of protein.

**Table 1**  
**Partial Purification of ACC Synthase**  
**from Cucurbita Tissue Slices<sup>a</sup>**

Step	Total Protein (mg)	Total Activity (nmol/h)	Specific Activity (nmol/h/mg protein)	Fold Purification	Recovery (%)
1. Crude Extract	17,600	105,000	6	1	100
2. Butyltoyo-pearl 650M	8,000	120,000	15	2.5	115
3. SP-Sephadex	800	49,000	62	10.4	47
4. QAE-Sephadex	336	45,800	136	23	44
5. Butyltoyo-pearl 650M	67	13,500	201	34	13
6. Sephacryl S-300	22	28,300	1,286	214	27
7. Bio Gel-HT	2.2	20,230	9,195	1,550	19
8. Mono-Q	0.22	7,830	35,590	6,000	7.5

<sup>a</sup>Amount of Tissue: 10 kg

Tissue Treatment: IAA 0.5 mM + BA 0.1 mM + LiCl 50 mM + AOA 1 mM for 24 hr.

SDS-PAGE conducted on fractions 16 and 17 from the mono Q column, which have the highest ACC synthase activity, indicated that the protein was not completely pure. (See Figure 4) However, it was demonstrated that the ACC synthase activity resided in the 46 kd band. The electrophoresis was conducted by applying 7.5 ml of the eluted fractions mixed with an equal volume of 2 x SDS loading buffer to a 10% polyacrylamide gel and silver staining. To determine the band containing ACC synthase activity, similar gels were run wherein the gels were cut into 3 mm thick slices and the ACC synthase activity was determined in half the slices; the other half were stained with silver.

The purified ACC synthase was also subjected to size exclusion chromatography on Sephacryl S-300. In this protocol, the ACC synthase eluted as an 86 kd species. This suggests that the Cucurbita ACC synthase consists of two identical 46 kd subunits. Further characterization showed that the pH optimum for ACC synthase activity is 9.5, and the isoelectric point is estimated at 5 using mono-P H 5/20 FPLC column chromatography. The  $K_m$  for AdoMet is 16.7 mM, and pyridoxal phosphate is a cofactor. The enzyme is stable at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  for over a year.

#### Example 3

##### Isolation of Zucchini Genomic Clones Encoding ACC Synthase

Four-day-old etiolated frozen zucchini seedlings were homogenized in 15% sucrose, 50 mM Tris-HCl, pH 8.5, 50mM EDTA- $\text{Na}_2$ , 0.25M NaCl. The nuclei were pelleted by centrifugation at 4,000 rpm for 10 min at  $4^{\circ}\text{C}$  and nuclear DNA was isolated by CsCl ethidium bromide equilibrium density gradient centrifugation. The recovered DNA was partially digested with Sau3A and electrophoretically separated on 0.5% low melting agarose.

DNA corresponding to 20 kb in size was ligated into EcoRI/BamHI cut EMBL 3 $\lambda$  (Frischauf, A.M., et al., J Mol Biol (1983) 170:827-842; Raleigh, E.A., et al., Proc Natl Acad Sci USA (1986) 83:9070-9074; Maniatis, T., et al.,

Molecular Cloning (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The ligation mixture was packaged and the library was screened without amplification by plating on E. coli strain K802 and screening with nick translated ACC1 cDNA (the full length zucchini cDNA clone) as described by Benton, D., et al., Science (1977) 196:179-183. The isolated genomic sequences were mapped with restriction endonucleases and the appropriate DNA fragments which hybridize to the ACC1 cDNA were subcloned into the pUC18 and pUC19 plasmids.

The results after restriction analysis of the various genomic clones recovered is shown in Figure 5A-C. As shown in the figure, two genomic clones reside on the same DNA strand, but are oriented in opposite directions. CP-ACC 1A and CP-ACC 1B each contain four introns. The complete genomic sequences of these clones are shown in Figures 6 and 7 respectively. As shown in Figures 6 and 7, the entire upstream regulatory sequences are encoded in the clones.

#### Example 4

##### Retrieval of Tomato cDNA Encoding ACC Synthase

Lycopersicon esculentum c.v. Rutgers was grown from seeds throughout the year in a greenhouse using protocols to ensure freedom from tobacco mosaic virus. The fruit was frozen and total RNA was isolated using the procedure of Chomczynski, P., et al., Anal Biochem (1987) 162:156-159. Approximately 5 gm of powdered frozen fruit tissue were used. Poly (A)<sup>+</sup> RNA was isolated using oligo (dT) cellulose chromatography as described by Theologis, A., et al. J Mol Biol (1985) 183:53-58, and a cDNA library was constructed into  $\lambda$ gt10 as described by Huynh, T.V., et al. cDNA Cloning Techniques: A Practical Approach (1985) (Glover, D.M. ed.), IRL Press, London, 49-78. cDNAs greater than 500 bp were inserted into the EcoRI site of the C1 repressor gene. The packaged DNA was plated on C600 HFL, a derivative of C600, to select for phage-containing inserts.

Approximately  $10^6$  plaque forming units of the  $\lambda$ gt10

recombinant phage were plated to a density of  $1 \times 10^4$  per 85 mm petri dish using C600. After transferring to nitrocellulose filters as described above, prehybridization and hybridization were performed at 37°C with gentle agitation in 30% formamide, 5X SSPE (1X SSPE is 0.1 M NaCl, 10 mM sodium phosphate, pH 7.0, 1 mM sodium EDTA), 5X BFP (1X BFP is 0.02% w/v bovine serum albumin, 0.02% polyvinyl pyrrolidone ( $M_r=360$  kd), 0.02% Ficoll ( $M_r=400$  kd), 100 mg/ml heat denatured salmon sperm DNA, and 0.1% SDS).

The gel purified 1.8 kb EcoRI fragment of the zucchini pACC1 prepared in Example 1 was labeled to a specific activity of  $2 \times 10^5$  cpm/mg using random hexamer printing and  $\alpha$ - $^{32}$ P dCTP as described by Feinberg, A.P., et al., Anal Biochem (1983) 132:6-13. The labeled probe was separated from starting material and used to probe the  $\lambda$ gt10 library.

The probe was denatured with 0.25 volumes 1M NaOH for 10 minutes at room temperature and neutralized with 0.25 volume 2M Tris HCl, pH 7.2 and then added to the hybridization mixture at  $1 \times 10^6$  cpm/ml.

After 24 hr hybridization, the filters were washed once in 30% formamide, 5X SSPE, 0.1% SDS at 37°C for 20 min and then four times in 2X SSPE, 0.1% SDS at 37°C for 20 min. The final wash was in 2X SSPE at 50°C for 10 min.

After washing, the filters were air dried, covered with Saran wrap and exposed at -70°C to X-ray film.

Using this hybridization, a full length cDNA from tomato, designated ptACC1, was recovered. The complete cDNA sequence of the ACC1 of tomato, designated ptACC1, is shown in Figure 8. Additional clones were recovered using  $2 \times 10^6$  cpm of the labeled 0.55 kb HindIII/EcoRI fragment at the 3' end of ptACC1.

Hybridization conditions were employed using  $2 \times 10^5$  pfu of the  $\lambda$ gt10 library on C600 wherein nitrocellulose platelets were prehybridized at 42°C and 50% formamide, 5X SSPE, 5X BPF, 500 mg/ml heat denatured salmon sperm DNA for 12 h. The filters were then hybridized for 18 hr at

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42°C with probe in 50% formamide, 5 X SSPE, 1X BFP, 100 mg/ml heat denatured salmon sperm DNA with this probe. The filters were washed at 42°C twice for 30 min in 50% formamide, 5X SSPE, 0.2% SDS, and then twice for 30 min in 0.1X SSPE at 42°C. A number of additional clones were retrieved using the above referenced portion of ptACC1 as shown in Figure 9.

A comparison of the amino acid sequences of the zucchini and tomato cDNA encoded ACC synthases is shown in Figure 10. As shown, considerable homology exists between these sequences, but they are not identical.

#### Example 5

##### Recovery of Tomato Genomic DNA Encoding ACC Synthase

Genomic DNA was isolated from etiolated Rutgers seedlings using a modification of the method described by Davis, R.W., et al. Meth Enzymol (1980) 65:404-411. Briefly, seedlings were grown on moist filter paper in the dark for 5 days at 22°C. Fifty g frozen hypocotyl and cotyledon tissue, seed coat removed, was ground in a coffee grinder. The powdered tissue was added to 200 ml of ice cold extraction buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaEDTA, 0.25 M NaCl, 15% sucrose (w/v)) and homogenized on ice using a hand held glass-glass homogenizer. The nuclei were pelleted at 2000 x g for 10 min at 4°C. The crude nuclei were resuspended in 100 ml of cold extraction buffer without the salt. To lyse the nuclei, 10 ml of 10% Nasarcosine was added, the suspension was gently inverted and incubated on ice for 10 min, then 120 g of CsCl was added and dissolved by gently shaking. To remove debris the solution was centrifuged at 26,000xg for 20 min at 4°C and the supernatant was decanted through Miracloth.

Ethidium bromide (10 mg/ml) was added to a final concentration of 0.4 mg/ml and the density of the solution was adjusted to 1.55 g/ml. Equilibrium centrifugation was carried out in a Beckman Ri70 rotor at 40,000 rpm for 48 hr at 20°C. The DNA was further purified by a second round of equilibrium centrifugation in a VTi65 rotor at 50,000 rpm for 16 hr at 20°C. Ethidium bromide was



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extracted from the DNA with isopropanol saturated with water containing 5 M NaCl and the DNA was dialyzed twice against 5000 volumes of TE (pH 7.5) to remove the CsCl. The typical yield was 15 µg/g fresh weight tissue.

5 Two genomic libraries were constructed, one with 15-23 kb Sau3A partially digested DNA in λEMBL3 and another with 6-8 kb DNA after complete HindIII digestion into λ2001. For the library constructed in λEMBL3, 100 µg of genomic DNA was digested with 1.5 units of Sau3A at 37°C  
10 in 300 µl of medium salt buffer (MSB) plus 2 mM dithiothreitol (DTT) (1X MSB is 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgSO<sub>4</sub>). One third of the reaction was removed at 7.5 min, at 10 min and at 12.5 min. At each  
15 time point digestion was stopped by adding 0.1 volume 0.5 NaEDTA, pH 8.0 and storing on ice. The DNA was size fractionated in a 0.5% low melting temperature agarose gel by electrophoresis at 0.8 Volts/cm for 24 h. The agarose gel electrophoresis buffer was 1X TAE, 40 mM Tris-HOAc, pH 8.0, 2 mM NaWDTA. The gel was soaked at room temperature  
20 for 3 hr in 1X TAE buffer containing 0.3 M NaCl. DNA was visualized with 365 nm ultraviolet light and the 15-23 kb side range was excised. The agarose was melted at 65°C for 15 min and extracted twice the TE (pH 7.5)-saturated phenol, prewarmed to 37°C. The aqueous phase was  
25 extracted three times with ether and two volumes of EtOH were added. After overnight at -20°C the DNA was collected by centrifugation and dissolved in TE, pH 7.5. Two µg of EMBL3 arms and 2µg size selected DNA were combined in a final volume of 6 µl, 1 µl of 10X ligase buffer  
30 (1X ligase buffer is 66 mM Tris-HCl, pH 7.5 5 mM MgCl<sub>2</sub>) was added and the cohesive ends annealed at 42°C for 15 min. The mixture was quickly cooled on ice and 1 µl each of 10 mM ATP and 50 mM DTT was added. The reaction was initiated with 1 µl (8 units) of T4 DNA ligase and  
35 incubated overnight at 14°C. One third of the ligation mix was packaged with Gigapak Gold (Stratagene) according to the manufacturer. Approximately 1 x 10<sup>6</sup> pfu were obtained when titered on C600.

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For the HindIII library, 200  $\mu$ g of genomic DNA was digested in 3.6 ml 1X MSB with 400 units of enzyme for 4 hr at 37°C. The DNA was separated on a 0.08% low melting temperature agarose gel and DNA in the 6-8 kb size range was isolated as described above. One  $\mu$ g of this DNA was ligated to 0.5  $\mu$ g of  $\lambda$ 2001 arms as described above in a final volume of 10  $\mu$ l. One third of this ligation was packaged with Gigapak Gold and  $5 \times 10^4$  pfu were obtained when plated on K802.

A BglII complete digest library and an MboI partial digest library of genomic DNA from tomato cultivar VF36, both in EMBL4, were provided by C. Corr and B. Baker. These libraries and the HindIII complete digest library in  $\lambda$ 2001 were plated on the host K802 and probed at high stringency with the ptACC1 cDNA as described above to obtain clones corresponding to the cDNA. Clones corresponding to other genes were obtained by probing the Sau3A partial digest library in EMBL3 with the cDNA at low stringency. In two separate screenings, phage were plated on the hosts C600 or TC410, lifted and fixed to nitrocellulose filters as described above. Low stringency prehybridization and hybridization were done in 30% formamide, 5X SSPE, 5X BFP, 100  $\mu$ g/ml denatured salmon sperm DNA, 0.2% SDS at 37°C for 18 h each. Probe was used at a concentration of  $10^6$  cpm/ml. Washing was done twice for 20 min in 30% formamide, 5X SSPE, 0.2% SDS at 37°C, and twice for 20 min in 2X SSPE, 0.2%, SDS at 42°C. The filters were exposed to X-ray film as above for 48 h.

For restriction enzyme digestions of  $\lambda$  clones, 2.5  $\mu$ g of phage DNA was digested in 50  $\mu$ l of high salt buffer (HSB) (1X HSB is 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgSO<sub>4</sub>) with the appropriate enzyme(s). For genomic DNA gel blots, 7.15  $\mu$ g of genomic DNA was digested in 100  $\mu$ l of 1X HSB with 80 units of EcoRI and HindIII or 40 units of BglII, at 37°C for 6 h. Digested DNAs were loaded on 1 cm thick, 0.8% agarose gels and electrophoresed at 3 V/cm in 1X TAE buffer containing 0.5  $\mu$ g/ml ethidium bromide. After electrophoresis the gel was photographed, the DNA

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42°C with probe in 50% formamide, 5 X SSPE, 1X BFP, 100 mg/ml heat denatured salmon sperm DNA with this probe. The filters were washed at 42°C twice for 30 min in 50% formamide, 5X SSPE, 0.2% SDS, and then twice for 30 min in 0.1X SSPE at 42°C. A number of additional clones were retrieved using the above referenced portion of pTACC1 as shown in Figure 9.

A comparison of the amino acid sequences of the zucchini and tomato cDNA encoded ACC synthases is shown in Figure 10. As shown, considerable homology exists between these sequences, but they are not identical.

#### Example 5

##### Recovery of Tomato Genomic DNA Encoding ACC Synthase

Genomic DNA was isolated from etiolated Rutgers seedlings using a modification of the method described by Davis, R.W., et al. Meth Enzymol (1980) 65:404-411. Briefly, seedlings were grown on moist filter paper in the dark for 5 days at 22°C. Fifty g frozen hypocotyl and cotyledon tissue, seed coat removed, was ground in a coffee grinder. The powdered tissue was added to 200 ml of ice cold extraction buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaEDTA, 0.25 M NaCl, 15% sucrose (w/v)) and homogenized on ice using a hand held glass-glass homogenizer. The nuclei were pelleted at 2000 x g for 10 min at 4°C. The crude nuclei were resuspended in 100 ml of cold extraction buffer without the salt. To lyse the nuclei, 10 ml of 10% Nasarcosine was added, the suspension was gently inverted and incubated on ice for 10 min, then 120 g of CsCl was added and dissolved by gently shaking. To remove debris the solution was centrifuged at 26,000xg for 20 min at 4°C and the supernatant was decanted through Miracloth.

Ethidium bromide (10 mg/ml) was added to a final concentration of 0.4 mg/ml and the density of the solution was adjusted to 1.55 g/ml. Equilibrium centrifugation was carried out in a Beckman Ri70 rotor at 40,000 rpm for 48 hr at 20°C. The DNA was further purified by a second round of equilibrium centrifugation in a VTi65 rotor at 50,000 rpm for 16 hr at 20°C. Ethidium bromide was

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was nicked with two 15 min treatments of 0.25 M HCl, denatured with two 20 min treatments of 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl and neutralized with two 20 min treatments of 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl. The nucleic acids were transferred in 20X SSPE to a Nytran nylon membrane.

After transfer was complete the nucleic acids were fixed with 1.2 joules of 254 nm ultraviolet radiation. The membranes were prehybridized in 50 ml of 50% formamide, 5X SSPE, 5X BFP, 1.0% SDS and 100 µg/ml heat denatured salmon sperm DNA at 42°C for 12 h. Hybridizations were carried out in 30 ml of 50% formamide, 5X SSPE, 1X BFP, 10% dextran sulfate ( $M_r=400$  kd), 0.2% SDS, and 50 µg/ml heat-denatured salmon sperm DNA at 42°C for 18 h. Filters with genomic DNA were hybridized with  $2.0 \times 10^6$  cpm/ml, whereas filters with  $\lambda$  DNA were hybridized with  $5 \times 10^5$  cpm/ml of random hexamer labeled 1.8 kb ptACC1 cDNA. After hybridization the membranes were washed two times for 20 min at 55°C in 0.1X SSPE and 0.2% SDS, dried, wrapped in plastic wrap and placed under Kodak XR-5 X-ray film.  $\lambda$  DNA gel blots were exposed for 12-24 hr at -70°C with an intensifier screen.

The clones corresponding to four different genomic clones recovered from tomato are shown in Figures 11 and 12. Figure 11A shows a series of three genomic clones which were identified to three separate genes LE-ACC 1A; LE-ACC 1B; and LE-ACC 3; Figure 12 shows genomic clones which were identified with LE-ACC 2. Figure 13 shows the complete nucleotide sequence of LE-ACC 2.

Figure 14 compares the amino acid sequences encoded by the six genomic clones recovered--two from zucchini and four from tomato. Again, conserved sequences are found and there is considerable homology; however, there are numerous differences in sequence.

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#### Example 6

##### Expression of Zucchini and Tomato cDNA in E. coli

The pACC1 from zucchini was subcloned into the EcoRI site of the expression vector pKK223-3 (DeBoer, H.A., et

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al, Proc Natl Acad Sci USA (1983) 80:21-25) and introduced into E. coli stain JM107. Transformants were grown in LB medium in the presence of ampicillin (50 mg/ml) at 37°C for 4 h. IPTG was added to 1 mM and the cultures were incubated for 2 hr at 37°C. ACC synthase activity and ACC formation were assayed. When the 1.7 kb EcoRI cDNA fragment was inserted into pKK2233-3 in the correct orientation and the transformed E. coli incubated as described above, ACC synthase activity was produced in the absence of IPTG at 20 nmol/h/mg protein and in presence of IPTG at 42 nmol/h/mg. ACC formation per 100 ml of culture was 2280 nmol without IPTG and 4070 nmol in the presence of IPTG. No ACC synthase activity or ACC production were observed when the 1-7 kb fragment was inserted in the opposite orientation.

A similar construct for tomato ACC synthase was used to test expression of the tomato cDNA in E. coli. The protein is synthesized as a fusion with a portion of the LacZ gene. The sequence at the junction is shown in Figure 15.

For construction of the vector containing this junction, pETC3C (Rosenberg, A.H., et al. Gene (1987) 56:125-155) was modified by cutting with EcoRI and EcoRV and filling in with Klenow to remove a 375 bp fragment downstream of the T7 promoter. The resulting religated plasmid was named pP07. pP07 was cut with BamHI and NdeI and the large DNA segment was purified and ligated to a BamHI/NdeI polylinker containing an EcoRI site to obtain the intermediate plasmid pP09.

The 1.4 kb EcoRI fragment from pTACC1 was then ligated into the EcoRI site of pP09 to obtain the junction shown in Figure 15 and designated pP046.

This plasmid was then used to transform E. coli BL21 (DE3) (Rosenberg et al. (supra)). The cultures were induced by diluting fresh overnight cultures into 2x TY (Maniatis et al. (supra)) and grown at 37° to an absorption at 600 nm of 0.7-0.8. IPTG was added to a final concentration of 2 mM and growth was continued for another

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two hours. The cells were harvested and the recombinant polypeptide was purified as described by Nagai, K. and Thogersen, A.C., Meth Enzymol (1987) 153:461-481.

Figure 16 shows the synthesis of ACC synthase in nmol/15  $\mu$ l of culture transformed with the tACC-containing vector in the presence and absence of IPTG. As shown in the figure, when the cDNA is ligated in the antisense direction, no ACC synthase is produced either in the presence or absence of IPTG (solid squares). When the cDNA is oriented in the correct orientation, after 180 min, over 2 nmol ACC synthase are produced after 15  $\mu$ l in the presence of IPTG (solid circles), and between 0.5 and 1.0 nmol. in the absence of IPTG (open circles).

The production of ACC using labeled  $C^{14}$ -carboxyl-labeled S-adenosyl-methionine is shown in Figure 17. In these figures, #1 is methionine, #2 is methionyl sulfite, #3 is methionyl sulfoxide, and #4 is unidentified. ACC is clearly labeled. Figure 17A shows the results in the absence of IPTG; a little ACC is formed. Figure 17C shows the results when the cDNA is ligated in the wrong orientation; no ACC is formed. Figure 17B shows the production of labeled ACC when the correct orientation of the cDNA is used. A large quantity of ACC is produced.

#### Example 7

##### Expression of Zucchini ACC Synthase in Yeast

The EcoRI fragment representing cDNA clone ACC1 was subcloned into the EcoRI site of the yeast expression vector pBM258 (Johnston, M., et al. Mol Cell Biol (1984) 4:1440-1448) and introduced into yeast strain YM2061. The yeast cells were grown on YP medium (Sherman, F., et al., Methods in Yeast Genetics (1979) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) at 37°C for 24 hr. The medium either contained 2% galactose or 2% glucose. After this culture, the cells were harvested and the supernatant was assayed for ACC released into the medium. The pelleted cells were resuspended in buffer containing 5 gm glass beads and vortex-mixed 10 times for 30 sec and centrifuged at 2000 x g for 3 min. This supernatant was

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also collected. Solid ammonium sulfate was added to achieve 80% saturation and the precipitate was collected and dissolved in 2 ml of 20 mM Tris-HCl, pH 8.0, 10  $\mu$ M pyridoxal phosphate, 10 mM EDTA, 0.5 mM dithiothreitol; and dialyzed against the same buffer. No ACC was produced in medium containing 2% glucose regardless of the construction of the vector. Control host vector and control vector with the ACC1 cDNA inserted in the anti-sense direction also gave no production of ACC in the cellular extract. However, when the medium contained 2% galactose, the pBM-ACC1 vectors containing the cDNA in the correct orientation did show the production of ACC in the crude extract as well as ACC activity in the extracted protein. ACC synthase activity was 2.6 nmol/hr/mg protein in the crude extract; 354 nmol of ACC were formed per 100 ml of culture.

#### Example 8

##### Antisense Inhibition of Ethylene Production in Tomato Plants

The ripening of tomatoes was shown to be preventable by the transformation of tomato plants with an antisense construction of the tomato ACC synthase gene which, therefore, putatively inhibited the synthesis of indigenous ACC synthase. The cDNA clone was inserted in the opposite sense direction under the control of the cauliflower CAMV 35S promoter and used to transform tomato plantlets using the A. tumefaciens mediated method. The regenerated plants produced tomatoes which failed to ripen, and which produced no ethylene at the times after pollination wherein ethylene was produced in control plants.

The antisense vector was constructed as follows: the 35S promoter was obtained as a 302 bp fragment from pJ024D (Ow, D.W., Proc Natl Acad Sci USA (1987) 84:4870-4874). The plasmid pJ024D was digested with HindIII, treated with Klenow, and then cut with BamHI to isolate the 302 bp fragment using gel electrophoresis. This was ligated to 3.5 kb of tomato ACC synthase cDNA by excising the coding

sequence from ptACC1 by digestion with XbaI, filling with Klenow, and then cutting with BamHI. The two BamHI fragments were ligated and the resulting ligation transformed into E. coli strain DH5A for cloning. The recovered plasmid was named pP032.

The plasmid pP032 was partially digested with SacI and SalI and the digest was ligated into SalI/SacI digested pBI101 binary Ti vector (Clonetech). pBI101 further contains the NOS 3' terminating sequences, as shown in Figure 18. The resultant vector, designated pP035 was transformed into E. coli DH5A for cloning. The sequences at the junctions were verified by sequence analysis.

pP035 or a control vector without the ACC-synthase gene was purified and introduced into Agrobacterium strain LBA4404 by a standard procedure described briefly as follows: A. tumefaciens LBA-4404 (2 ml) was grown overnight at 28°C in LB broth, and this used to inoculate 50 ml of LB broth to obtain the desired culture. The inoculated medium was grown at 28°C until the OD<sub>600</sub> was 0.5 - 1.0. The cells were collected by centrifugation and the pellet was resuspended in 1 ml, 20 mM ice cold CaCl<sub>2</sub>. To 100 µl of the cell suspension, 1 µm of pP035 DNA was added, and the mixture was incubated on ice for 30 min before snap-freezing in liquid nitrogen. The cells were then thawed at 37°C for 5 min and used to inoculate 1 ml LB. After 2 h growth at 28°C with agitation, 100 µl of the culture were plated on LB+Kan<sub>50</sub> medium; colonies appeared in 2-3 days at 28°C. The cells were recultured by picking several colonies and streaking on LB+Kan<sub>50</sub> medium; again, 3-4 colonies were picked from independent streaks and 5 ml cultures in LB+Kan<sub>50</sub> medium were grown. Stationary phase of these cultures were used for transfection of tomato plants.

The cells can be frozen using 15% glycerol at -80°C to store for later use.

#### Preparation of Host Plants

Tomato seeds were sterilized using a protocol which





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consisted of treatment with 70% ethanol for 2 min with mixing; followed by treatment with 10% sodium hypochlorite and 0.1% SDS for 10 min with mixing, followed by treatment with 1% sodium hypochlorite, 0.1% SDS for 30 min with mixing, and washing with sterile water 3X for 2 min each wash.

For germination, 0.8 g of the sterilized seeds were placed in a Seed Germination Medium in a filled magenta box and grown for 2 weeks at low light in a growth room. The magenta box contained 30 ml of the medium<sup>1</sup>.

After two weeks, when the seeds had germinated, cotyledons were dissected for the seedlings by cutting off the cotyledon tips and then cutting off the stem. This process was conducted in a large petri dish containing 5-10 ml of MSO.<sup>2</sup>

The harvested cotyledons were placed abaxial side up in tobacco feeder plates and grown for 48 h.

The feeder plates were prepared from a tobacco cell suspension in liquid medium<sup>3</sup> at 25°C prepared with shaking at 130-150 rpm. The suspension was transferred to fresh medium at 1:10 dilution per every 3-5 days. 1 ml of rapidly dividing culture was placed on the feeder plate, overlaid with filter paper and placed in low light in a growth room. The feeder plates were supplemented with 10

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<sup>1</sup> Seed Germination Medium contains, per liter, 2.16 g of Murashige-Skoog salts; 2 ml of 500X B5 vitamins which had been stored at 20°C, 30 g sucrose and 980 ml water, brought to 1 N KOH and containing 8 g agar. The medium is autoclaved in 500 ml portions before filling the magenta boxes.

<sup>2</sup> MSO contains per liter 4.3 g of Murashige-Skoog salts, 2 ml of 500 X B5 vitamins; 30 g of sucrose and 980 ml of water made 1 N in KOH to a final pH of 5.8.

<sup>3</sup> Tobacco Suspension Medium contains in 1 liter 4.3 g Murashige-Skoog salts, 2 ml of 500X B5 vitamins, 30 g 3% sucrose, 10  $\mu$ l of a 0.5 mg/ml solution of kinetin stored at -20°C, 2 ml of a 2 mg/ml solution of pCPA, and 980 ml of water made 1 N in KOH for a pH of 5.8 and autoclaved in 50 ml portions per 250 ml flask.

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ml Feeder Medium.<sup>4</sup> The Agrobacterium containing the pP035 vector was inoculated into 50 ml LB containing kanamycin with a single colony of the strain. The culture was grown shaking vigorously at 30°C to saturation (OD>2.0 at 600 nm). The strain was chosen to come to full growth in less than 24 h. The culture was then diluted to  $5 \times 10^8$  cell/ml with MSO and split into 50 ml portions in plastic tubes.

Cotyledons from two of the feeder plates were scraped into each tube and rocked gently for 10-30 min. The cotyledons were then removed from the bacterial culture into sterile filter paper abaxial side up on a tobacco feeder plate and incubated for 48 h in low light in a growth room.

The cotyledons were then transferred axial side up to Callus Inducing Medium.<sup>5</sup>

In the Callus Inducing Medium, approximately four plates will be used per magenta box, and the explant are crowded. The box is placed in a growth room for three weeks, and small masses of callus formed at the surface of the cotyledons. The explants are transferred to fresh plates containing the callus inducing medium every three weeks.

When the calli exceeded 2 ml, they were transferred

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<sup>4</sup> Feeder Medium contains 0.43 g Murashige-Skoog salts, 2 ml 500X B5 vitamins, 30 g of sucrose and 980 ml water made 1 N in KOH to a pH of 5.8, including 0.8% agar. The foregoing components are autoclaved in two 500 ml portions and hormones are added when pouring plates to obtain 1  $\mu$ /ml benzyl adenine (BA) and 0.2  $\mu$ g/ml of indole acetic acid (IAA).

<sup>5</sup> Callus Inducing Medium contains per liter 4.3 g of Murashige-Skoog salts, 2 ml of 500X B5 vitamins, 30 g of sucrose and 980 ml of water brought to 1 N KOH at a pH of 5.8. The medium contains 0.8% agar and is autoclaved in two 500 ml portions. When poured into the plates, the following hormones are added to the following concentrations: 1  $\mu$ m/ml BA, 0.2  $\mu$ g/ml IAA, 100  $\mu$ g/ml kanamycin, 500  $\mu$ g/ml carbenicillin (Geopen).

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to plates containing shoot inducing medium.<sup>6</sup>

When the stem structure is evident, the shoots were dissected from the calli and the shoots were transferred to root inducing medium-containing plates.<sup>7</sup>

5 After a vigorous root system was formed on the plants, the plantlets were transferred to soil. To do this, they were taken from the plates, removing as much agar as possible and placed in a high peat content soil in a small peat pot which fits into a magenta box with  
10 cover. When the seedling leaves reached the top of the box, the lid was loosened and continued to be uncovered slowly over a period of 4-5 days. The plants were then transferred to a light cart and larger pots, and kept moist.

15 The regenerated tomato plants were allowed to flower and pollinated. Seeds from the regenerated plants were replanted and grown to maturity. Flowers of these first generation plants were pollinated and tomatoes were developed and ethylene measured by gas chromatography at  
20 specified days after pollination. Figures 19 and 20 show the results for two sets of individual plants VI.1-4 (which contains the control vector) and AII.2-24 (which contains the antisense vector) in Figure 19 and VI.1-6 (which contains the control vector) and AII.2-7 (which  
25 contains the antisense vector) in Figure 20. As shown in these figures, in both cases, the control plants which had

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<sup>6</sup> Shoot Inducing Medium contains, per liter, 4.3 g of Murashige-Skoog salts, 2 ml of 500X B5 vitamins, 0.6 g of MES and 900 ml of water made 1 N in KOH for a pH of  
30 5.8, and 0.8% agar. The medium is autoclaved in two 450 ml portions and then is added 100 ml of a 30% filtered, sterilized glucose solution. When the plates are poured, additional components are added as follows: 0.1 mg/ml zeatin, 100 µg/ml kanamycin, 500 µg/ml carbenicillin.

35 <sup>7</sup> Root Inducing Medium contains, per liter, 4.3 g Murashige-Skoog salts, 2ml 500X B5 vitamins, 30 g of sucrose and 980 ml of water, 1 N in KOH to a pH of 5.8 in 0.8% agar. The medium is autoclaved in two 500 ml portions and when pouring plates, hormones are added to a  
40 concentration of 100 µg/ml kanamycin and 500 µg/ml or carbenicillin.

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5 been transformed with control vector produced high levels of ethylene up to 8 ng/g fruit/h after approximately 50 days after pollination either in the presence of propylene or in the presence of air. However, in both cases, there was no production of ethylene in those plants which had been transformed with the antisense pP035 vector. In addition, the tomatoes which failed to produce ethylene also failed to ripen, whereas the control plants did ripen at this time.

The claims defining the invention are as follows:

1. A DNA molecule including an expression system that is capable of generating, when contained in a plant host cell, a complementary RNA that is sufficiently complementary to an RNA transcribed from an endogenous ACC synthase gene to prevent synthesis of said endogenous ACC synthase, wherein said expression system includes the reverse transcript of said complementary RNA, said reverse transcript being operably linked to control sequences that effect its transcription into said complementary RNA.
2. The DNA molecule of claim 1, wherein the endogenous ACC synthase gene is an ACC synthase gene of tomato or zucchini.
3. The DNA molecule of claim 2, wherein said ACC synthase gene is either LE-ACC2 or pACC1.
4. A plant or plant cell modified to contain the DNA molecule of any one of claims 1-3.
5. The plant or plant cell of claim 4 that is either tomato or zucchini.
6. A method for inhibiting ethylene production in a cell that normally produces ethylene, said method includes culturing *in vitro* or *in vivo* a cell that has been modified to contain the DNA molecule of any one of claims 1-3 under conditions wherein said complementary RNA is expressed.
7. The method of claim 6, wherein said cell is either a tomato cell or a zucchini cell.
8. The method of claim 6, wherein said complementary RNA complements RNA transcribed from either a tomato ACC synthase gene or zucchini ACC synthase gene.
9. A transgenic plant that exhibits properties resulting from a lack of sufficient endogenous ACC synthase, wherein one of said properties is a delay of fruit ripening and wherein said transgenic plant has been modified to contain a DNA molecule including an expression system that generates an RNA complementary to RNA transcribed from an endogenous ACC synthase gene.
10. The plant of claim 9, which is either a tomato or a zucchini plant.
11. The plant of claim 9, wherein said complementary RNA is complementary to RNA transcribed from either a tomato ACC synthase gene or a zucchini ACC synthase gene.



12. The plant of claim 9, wherein said ACC synthase gene is either LE-ACC2 or pACC1.

13. The plasmid designated as pP032.

14. A DNA molecule according to claim 1 substantially as hereinbefore described  
5 with reference to any one of the examples or accompanying drawings.

DATED this     day of     1994.

10 PHILLIPS ORMONDE & FITZPATRICK

ATTORNEYS FOR:  
OLIFF & BERRIDGE

*David B Fitzpatrick*

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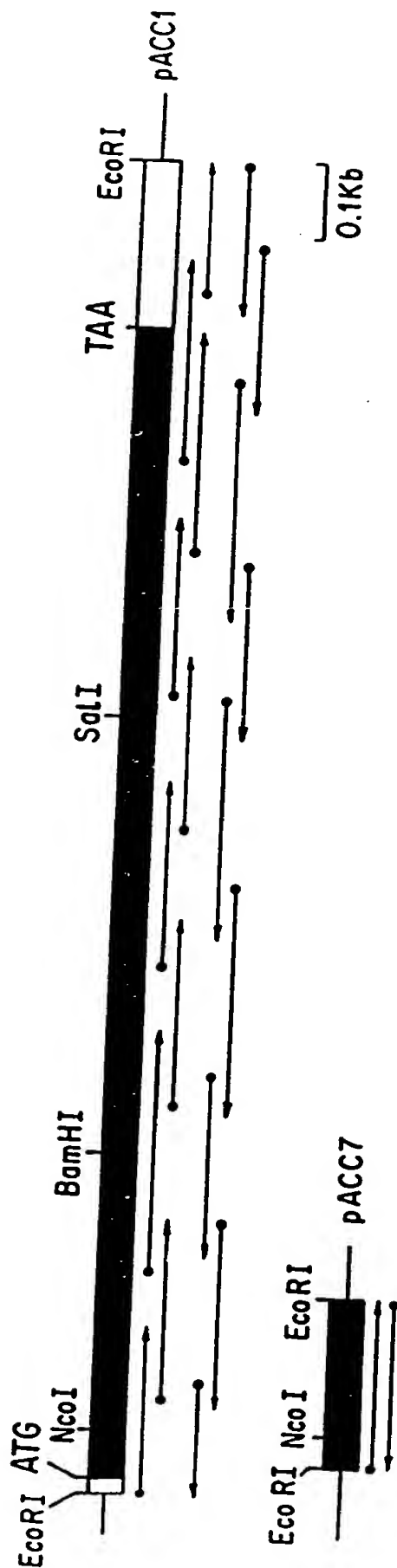


FIG. 1A

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   E R N Q A L  
 TCTCGAAGATCGCCCTCGACGATGGCCATGGCGAGAAGCTCCCGTATTTTC 100  
 L S K I A L D D G H G E N S P Y F  
 L S K I A L D D G H G E N S P Y F ← pACC7  
 GATGGGTGGAAAGCTTACGATAACGATCCGTTTCACCCTGAGAATAATCC  
   D G W K A Y D N D P F H P E N N P  
   D G W K A Y D N D P F H P E N N P  
 TTTGGGTGTTATTCAAATGGGTTTAGCAGAAAATCAGCTTTCTTTTGATA 200  
   L G V I Q M G L A E N Q L S F D  
   L G V I Q M G L A E N Q L S F D  
 TGATTGTTGACTGGATTAGAAAACACCCTGAAGCTTCGATTTGTACACCG  
 M I V D W I R K H P E A S I C T P  
 M I V D W I R K H P E A S I C  
 GAAGGACTTGAGAGATTCAAAAGCATTGCCAAGCTTCCAAGATTACCACGG 300  
   E G L E R F K S I A N F Q D Y H G  
 CTTACCAGAGTTTCGAAATGCAATTGCAAATTTTATGGGGAAAGTAAGAG  
   L P E F R N A I A N F M G K V R  
 GTGGGAGGGTAAAATTTCGACCCGAGTCGGATTGTGATGGGTGGCGGTGCG 400  
 G G R V K F D P S R I V M G G G A  
 ACCGGAGCGAGCGAAACCGTCATCTTTTGTGTTGGCGGATCCGGGGGATGC  
   T G A S E T V I F C L A D P G D A  
 TTTTTTGGTTCCTTCTCCATATTATGCAGGATTGTGATCGAGACTTGAAAT 500  
   F L V P S P Y Y A G F D R D L K  
 GGCGAACACGAGCACAAATAATTCGGGTCCATTGCAACGGCTCGAATAAC  
 W R T R A Q I I R V H C (N) G S N N  
 TTCCAAGTCACAAAGGCAGCCTTAGAAATAGCCTACAAAAAGGCTCAAGA 600  
   F Q V T K A A L E I A Y K K A Q E  
 GGCCAACATGAAAGTGAAGGGTGTATAATCACCAATCCCTCAAATCCCT  
   A N M K V K G V I I T N P S N P  
 TAGGCACAACGTACGACCGTGACACTCTTAAACCCCTCGTCACCTTTGTG 700  
 L G T T Y D R D T L K T L V T F V  
 AATCAACACGACATTCACTTAATATGCGATGAAATATACTCTGCCACTGT  
   N Q H D I H L I C D E I Y S A T V  
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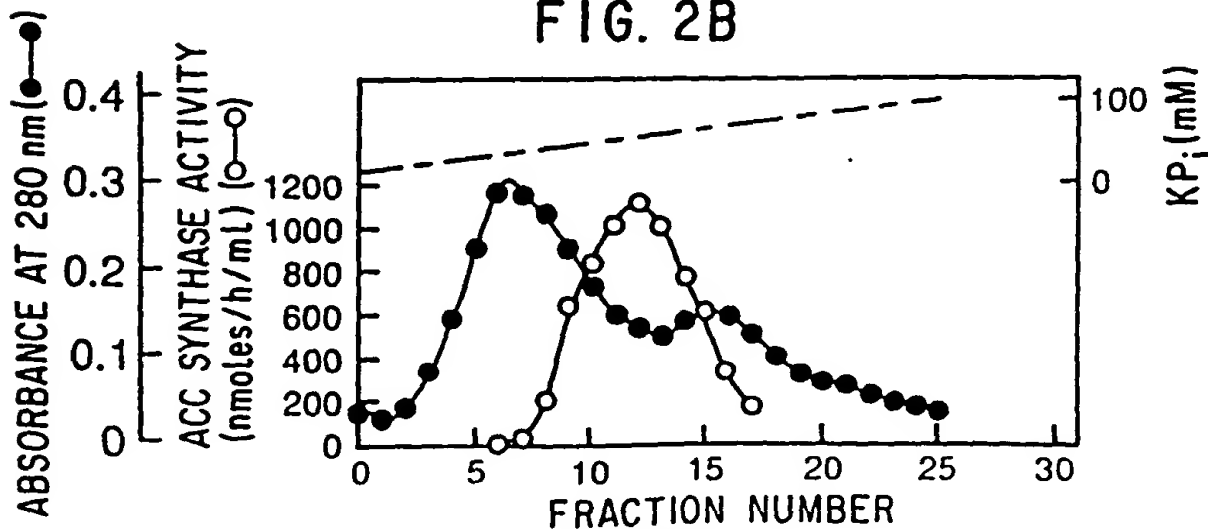
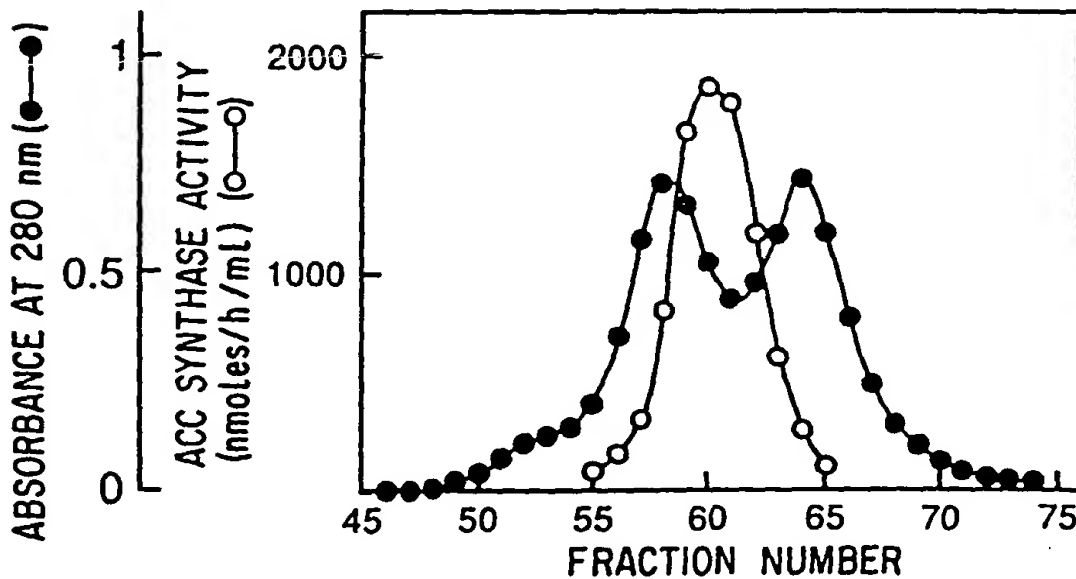
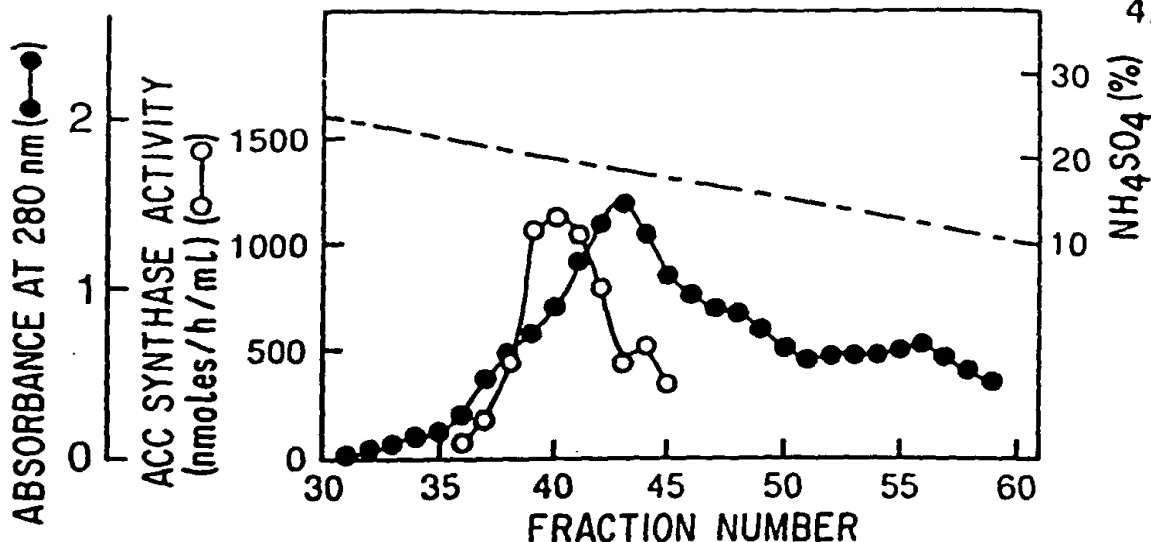
FIG. 1B(i)

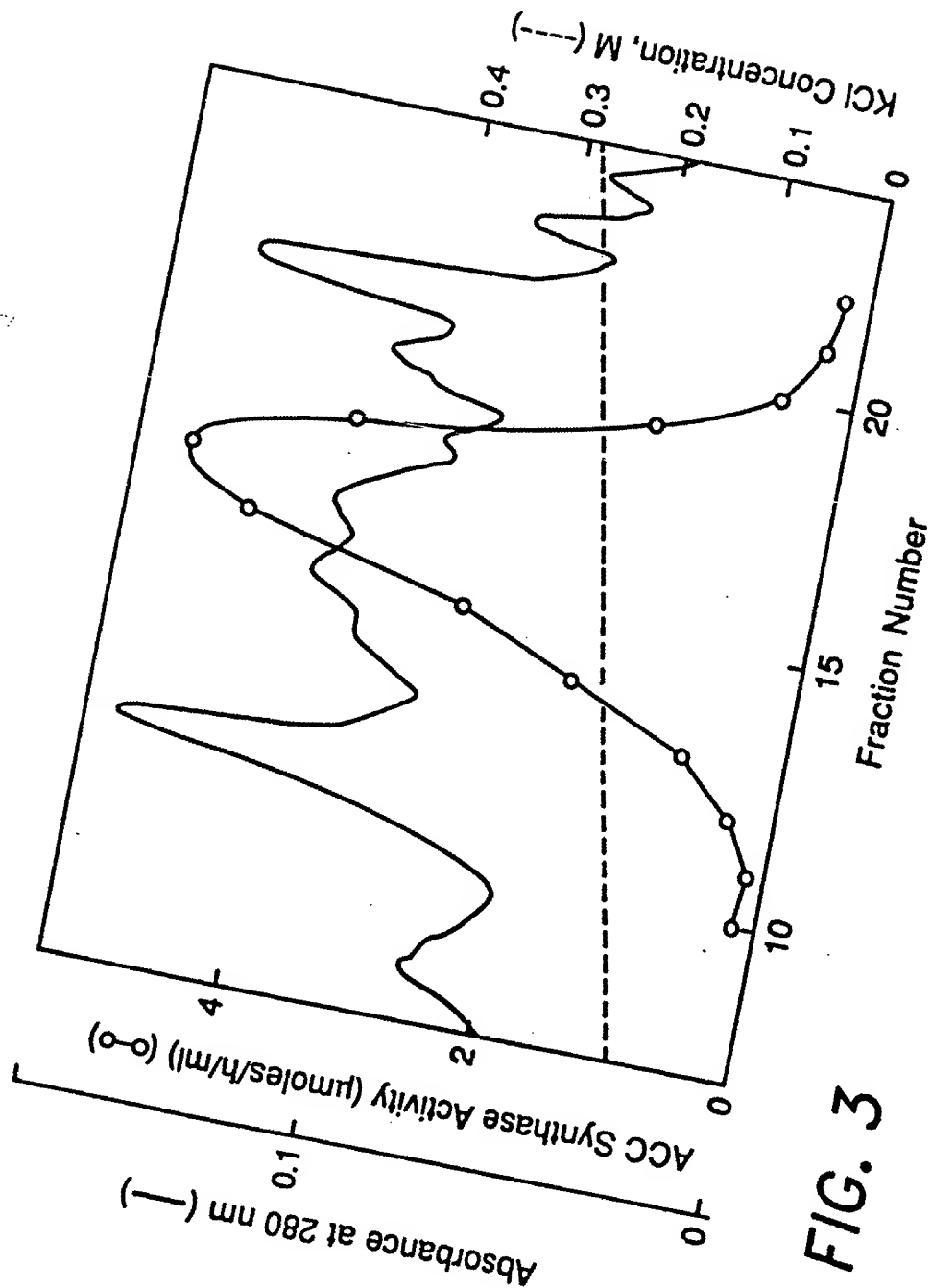
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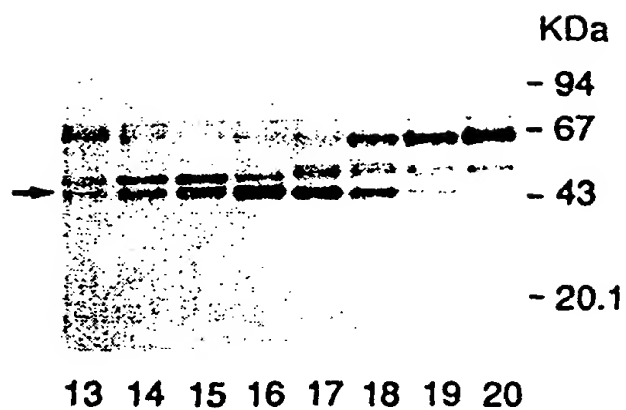


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 M G L P G F R V G I I Y S Y N D V  
 CGTCGTCCGCCGTGCTCGGCAGATGTGAGCTTCGGCCTCGTCTCGTCCC  
 V V R R A R Q M S S F G L V S S  
 AGACTCAACATTTGCTCGCCGCCATGCTTTCCGACGAGGACTTTGTGCGAC 1000  
 Q T Q H L L A A M L S D E D F V D  
 AAATTTCTTGCCGAGAAGCTCGAAGCGTGTGGGCGAGAGGCATGCAAGGTT  
 K F L A E N S K R V G E R H A R F  
 CACAAAAGAATTGGATAAAATGGGGATCACTTGCTTGAACAGCAATGCTG 1100  
 T K E L D K M G I T C L N S N A  
 GAGTTTTTGTGTGGATGGATCTACGGAGGCTATTAAAAGACCAAACCTTC  
 G V F V W M D L R R L L K D O T F  
 AAAGCTGAAATGGAGCTTTGGCGTGTGATTATCAATGAAGTCAAGCTCAA 1200  
 K A E M E L W R V I I N E V K L N  
 TGTTCCTCCTGGCTCATCCTTTCATGTCAGTGAAGCCAGGTTGGTTTCGAG  
 V S P G S S F H V T E P G W F R  
 TTTGTTTCGCAAACATGGACGACAACACCGTTGACGTTGCTCTCAATAGA 1300  
 V C F A N M D D N T V D V A L N R  
 ATCCATAGCTTTGTGCGAAAACATCGACAAGAAGGAAGACAATACCGTTGC  
 I H S F V E N I D K K E D N T V A  
 AATGCCATCGAAAACGAGGCATCGAGATAATAAGTTACGATTGAGCTTCT 1400  
 M P S K T R H R D N K L R L S F  
 CCTTCTCAGGGAGAAGATACGACGAGGGCAACGTTCTTAACCTACCGCAC  
 S F S G R R Y D E G N V L N S P H  
 ACGATGTCGCCTCACTCGCCGTTAGTAATAGCAAAAAATTAAtttaaaac 1500  
 T M S P H S P L V I A K N  
 atttttcaaaatattcataccattcatatagtttttttttttttttttttt  
 tgggtcaatgttgactaaagttacgtatatttttttccacagtggatatga 1600  
 tgtaaacttcatatttttttgggtgggatgggtgatagatgtaatgtatttgg  
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 tat

FIG. 1B(ii)





**FIG. 4****SUBSTITUTE SHEET**



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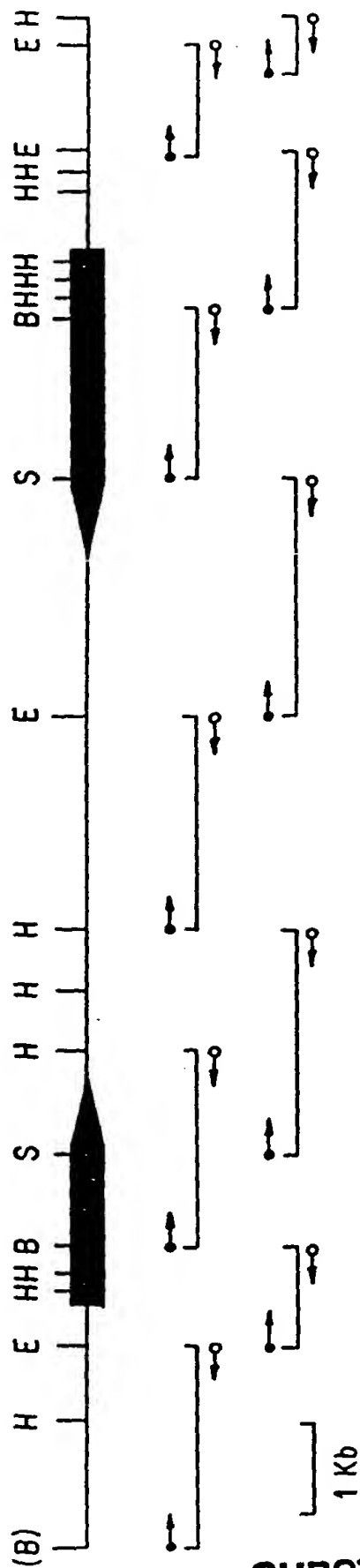


FIG. 5B

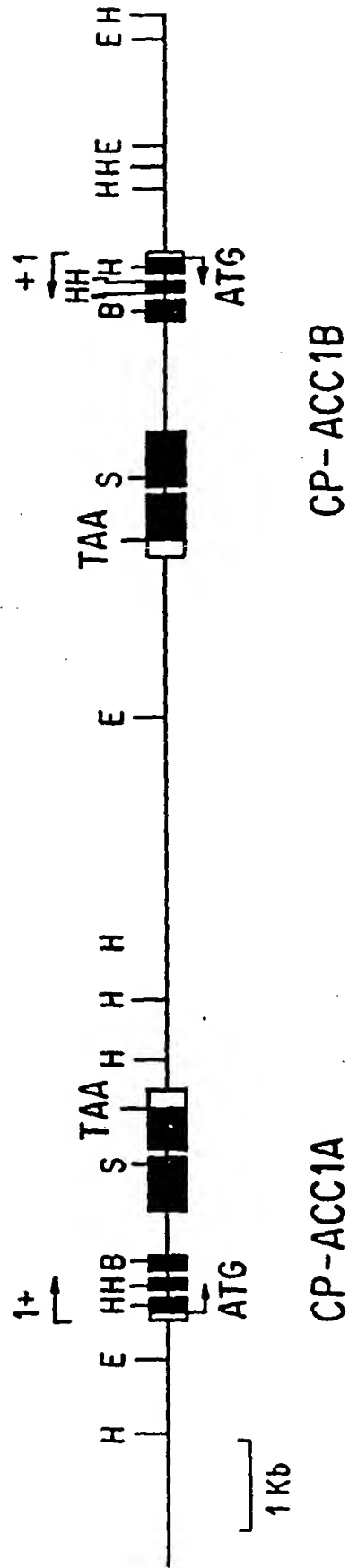


FIG. 5C

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ca

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 aaggggtggattgtgagatcccacgaacgaacattctttgttaaggggtg  
 gaaaacctctccctagcatactcgttttaaaaacctcaaggagaagtaca -2501  
 aaaagaaaagccgagggaaggatttcaaaaagttagaacttcatataaaat  
 gaaagcacaaagaaagagaattattagtaattgttcttgcacaagtataagt  
 tgaaaaacttaattctatcaagtgtgaatccacactcatctttcaaaaatta  
 agcaaacaaaaacgagtcattgcttgccttctccaaaattttatcactaatag  
 tgtgacactcaatgtcccacttaccattcttggcccccacaaccacctcc  
 aagaaacaaataacttttactacccaaccccccaattttggaaacaaaaatga  
 gtcaaatatatagaacaaataacatcgtgtttcttcttaccgactcggttga  
 atatgcaacgtttataatataacttcaagaaaattttgagacattactcaaa  
 taagictctcacaaaaatagaatatctttatactagtataatgaattgt  
 ccacttcgatttaaatcctctaaagttcactttcgtanaatggcttaatga -2001  
 acagatttattaggatcaaatcaaaaagttgaatgagactaaa tagatat  
 aataaaaatctgaattgttgcatgaagtatgcagctcaaaagatga tgttttg  
 cgaaaaaaaatgcaaaactaagcatgagtgcttctgtaaaaaaaataa gaaa  
 aagaaaaatatatactgtactatcaaaaacattgtccttacttagacagc  
 tcaaaaacttttcataattcctataatttgtttataattgaaactttttccatt  
 tcatttgttttaaatcataatttggttgttttaaatagaatactgtaacagt  
 ccaagctcactgttagtagatatgttcttcttccggacttttccggcttct  
 tctcaagggttttaaaaatgtgtctactagggagagattttcacacacttat  
 aaagaatgatctcgttctcttcaactaatgtanaaatctcacaaaatact  
 aaacaattggaaatttattaggatcagaatcaaaaagttgagagatatagt -1501  
 gaaacgaccgtcgagattaaatagatatcaaatcaagtttgatcattgtact  
 aaataagtagctcggagatgtatagcagaaaaagaaagcgactataaaaa  
 tgaggtaaaaagtggtcggagtagtatcaaatgtgagagggtatgcaaatat  
 tacgtatttcttttaggtgaaaaagttccgaaaccacaccaaaaagcactc  
 ttaaaaaatgtgccaaaacgggttctatcactcaatgtcaaatctttcaatt  
 caaaaagcatgtgggtattgattgcttgcctccaacgaagcttcatctcct  
 acttgttacacacacacaaaactcgttgttcatgaccaattctatccccct  
 tcccatgtcatcctccaaaacttttgacccttcaatttgggtccccctaacc  
 tttttttcatcacaaggatgcaaccattttgatttagtctacgacattc  
 ttttcatttatctacttacgcccctcggagggaacagttggattgaaagtt -1001  
 cgacttcttagccttggagatgagagaaccggtacactccatgaattaca  
 aattttaaaatctctaaatcctaactttggagctacgtatgacctttgtatc

FIG. 6A

INSTITUTE SHEET

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tttgtaagagcttttctcaatgctaacaaaatttgctctatttcagctgg  
 tacgcattgtcgtctgcttcccgaattttaaaatagctctattaaggagag  
 gttttcacacccttactagaaacgtttcgttctccctctaaatgtgagat  
 ctaccgtaactagctagagattaaaatgttattatagctagagattcaa  
 ccaaacataacacaaaaagataatcatagggatcaacaaaattcataact  
 agttcttataatatgcaataaaattcaaatataattatgcattagaagaaa  
 ataaaaaaacaattagaataacccaaaaattaatcttcttacctataa  
 atctataataagattcgagtattagattaaaattatcccaaatcaagaac -501  
 ataaattaagatcataaacgtaatatattttaatcgagaacgtaaataca  
 ggacatacagattagaattcaaaaatatttgaattataatagaatttga  
 tagaaaaataaaacaaaaactaaaaaaataaacttagtaattatgatgagat  
 aaaagaagattttgtgacatgatatattttgttatgttccaaatctagagt  
 atgcctccacacatgcggggcgggcgggctgtgtgtgtggctcgtctgc  
 ttgcttgaatcacaccctccacgcatgcaattacgccctccttgactca  
 aaccccattttaactctctcttccatttttattatttttctttaatttt  
 tttcatcactgtttttttttttttttttttcatggtttgaactttgaaa  
 agttgaattttctacacgttttgattttcctggtaagaacttgatcttgtt  
 ggatcttctcactgcttataaattcactcaattctcttctttctttcct -1  
 ATCTTACAACCCAAAACCTCTCATTTTTAGGCACATCTCAACAACCTTCA  
 AATGGGGTTTCATCAAATCGACGAAAGGAACCAAGCTCTTCTCTCGAAGA 100  
 M G F H Q I D E R N Q A L L S K  
 TCGCCCTCGACGATGGCCATGGCGAGAACTCCCGTATTTCGATGGGTGG  
 I A L D D G H G E N S P Y F D G W  
 AAAGCTTACGATAACGATCCGTTTCACCCTGAGAATAATCCTTTGGGTGT  
 K A Y D N D P F H P E N N P L G V  
 TATTCAAATGGGTTTAGCAGAAAATCAGgttttggtatatcgtgttttcgt  
 I Q M G L A E N Q  
 gtttttcttatatgacttcacgtttgaaaaatttcgctaactttgtttttt  
 tgtgaatttcgatagCTTTCCTTTGATATGATTGTTGACTGGATTAGAAA  
 L S F D M I V D W I R K  
 ACACCCTGAAGCTTTCGATTGTACACCGGAAGGACTTGAGAGATTCAAAA  
 N P E A S I C T P E G L E R F K  
 GCATTGCCAACTTCCAAGATTACCACGGCTTACCAGAGTTTCGAAATgta  
 S I A N F Q D Y H G L P E F R N

FIG.6B



11/40

cgagatatgatatactctttaactatatctgaactcaaaagggttaagttga 500  
 tgggttatgataaaatttctttcttgtagGCAATTGCAAATTTTATGGG  
 A I A M F M G  
 GAAAGTAAGAGGTGGGAGGGTAAATTCGACCCGAGTCGGATTGTGATGG  
 K V R G G R V K F D P S R I V M  
 GTGGCGGTGCGACCGGAGCGAGCGAAACCGTCATCTTTTGTGGCGGAT  
 G G G A T G A S E T V I F C L A D  
 CCGGGGGATGCTTTTTTGGTTCCTTCTCCATATTATGCAGGgtgagttct  
 P G D A F L V P S P Y Y A G  
 tctttcatttcttttgttcacttttctttaagtcatttcttagtcc  
 aacctggaaagagaaagaagagagagaaagaaaccatttgacaaattaat  
 aactctacaaattctctttgaaagtttgatgtttttttaaggtaaaac  
 ttcaaccattctcttgcaaaagaaaaaaagtcataattataatgaaga  
 aaaaactaggccatccaagtcaacctttttaaatgctaataaagtcaata  
 tgctttgtaggtttaaaaaacaataaattgcttaatcatttcttaaat 1000  
 taattaaaccttttgaactttatcattaccatttacataaatttaacaat  
 ttattgctctttttgtagtataaataaaaaaaagttagggtgtaaac  
 gtacagtattatgttattgtataaataactgagaagtgtagtatgttgtt  
 tttcagATTTGATCGAGACTTGAAATGGCGAACACGAGCACAAATAATTC  
 F D R D L K W R T R A Q I I  
 GGGTCCATTGCAACCGCTCGAATAACTTCCAAGTCACAAAGGCAGCCTTA  
 R V H C N R S N N F Q V T K A A L  
 GAAATAGCCTACAAAAGGCTCAAGAGGCCAACATGAAAGTGAAGGGTGT  
 E I A Y K K A Q E A N M K V K G V  
 TATAATCACCAATCCCTCAAATCCCTTAGGCACAACGTACGACCGTGACA  
 I I T N P S N P L G T T Y D R D  
 CTCTTAAACCCCTCGTCACCTTTGTGAATCAACACGACATTCACTTAATA  
 T L K T L V T F V N Q H D I H L I  
 TGCGATGAAATATACTCTGCCACTGTCTTCAAAGCCCCAACCTTCACCAG  
 C D E I Y S A T V F K A P T F T S  
 CATCGCTGAGATTGTTGAACAAATGGAGCATTGCAAGAAGGAGCTCATCC 1500  
 I A E I V E Q M E H C K K E L I  
 ATATTCTTTATAGCTTGTCCAAAGACATGGGCCTCCCTGGTTTTTCGAGTT  
 H I L Y S L S K D M G L P G F R V  
 GGAATTATTTATTCTTACAACGATGTGCTCGTCCGCCGTGCTCGGCAGAT  
 G I I Y S Y N D V V V R R A R Q M  
 GTCGAGCTTCGGCCTCGTCTCGTCCCAGACTCAACATTTGCTCGCCGCCA  
 S S F G L V S S Q T Q N L L A A

FIG.6C

SUBSTITUTE SHEET

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TGCTTTCCGACGAGGACTTTGTGACAAATTTCTTGCCGAGAACTCGAAG  
M L S D E D F V D K F L A E N S K  
CGTGTGGGCGAGAGGCATGCAAGgtttgttaaactacaccattattttt  
R V G E R H A R  
gtgggattgaaaagcattactttttgcaattaatttaagaatgtattaat  
caaattcagGTTACAAAAGAATTGGATAAAATGGGGATCACTTGCTTGA  
F T K E L D K M G I T C L  
ACAGCAATGCTGGAGTTTTTGTGTGGATGGATCTACGGAGGCTATTAAAA  
N S N A G V F V W M D L R R L L K  
GACCAACCTTCAAAGCTGAAATGGAGCTTTGGCGTGTGATTATCAATGA  
D Q T F K A E M E L W R V I I N E  
AGTCAAGCTCAATGTTTCTCCTGGCTCATCCTTTCATGTCACTGAGCCAG  
V K L N V S P G S S F H V T E P  
GTTGGTTTCGAGTTTGTTCGAAACATGGACGACAACACCGTTGACGTT  
G W F R V C F A N M D D N T V D V  
GCTCTCAATAGAAATCCATAGCTTTGTGAAAACATCGACAAGAAGGAAGA  
A L N R I H S F V E N I D K K E D  
CAATACCGTTGCAATGCCATCGAAAACGAGGCATCGAGATAATAAGTTAC  
N T V A M P S K T R H R D N K L  
GATTGAGCTTCTCCTTCTCAGGGAGAAGATACGACGAGGGCAACGTTCTT  
R L S F S F S G R R Y D E G N V L  
AACTACCGGCACACGATGTCGCCTCACTCGCCGTTAGTAATAGCAAAAAA  
N S P H T M S P H S P L V I A K N  
TTAATTA AAAACATTTTTTCAAATATTCATACCATT CATATAGTTTTTTT

2000

\*

TTTTTTTTTTTTTTGGGTCAATGTTGACTAAAGTTACGTATATTTTTTCC  
ACAGTGGATATGATGTAAACTTCATATTTTTTGGTGGGATGGTGATAGAT  
GTAATGTATTTGGTTTTTCCCTTAGGGAACCTACTTATTTATTAATGA  
AATGATTGTGATTTATGAattataattgtatatttttctttaaaagtatt  
ttattgcaaaaaataaataagttattatgaggaattgtaattgaaatggaaaa  
ggtatagagtcaaagggaataaacatatattttattttttcttatggaaag  
ttttgttcatacttaaaaatgtattatatttatggaaactttattgacttt  
aaagattttgggacaaagggtatgatatgttcaagtttattacgtttgttg  
gattagtcacttcattgacattgatgtttttgttgatcatattttgtcatt  
attaccacactttttttgtctaaaagcaagcttataattcaatgaggatgc  
aaaaatactttataaatggtttgtctatgtttgggtctcatagatgcacc  
tttatacaaaaccgttcatacaaaaccaaattataatgtcgaatccag  
aaacgctatatatacaaaagtcaaatactttactgacaaactacgatcgttca

2500

FIG. 6 D

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ccgtcctataacatctttttcgagtgctaacattcaa tgttacatcgttt 3000  
tttttttttgtttggtaaa tacttttttcttttgctttgttaaattataa  
cttgggtttgttatgtgcaatttatctattatata tgcagttgacttagtt  
agcttgattgttctagtagtgaa tgaactagta tcttgagttgaggggct  
acctcataaaa tctagtaggacgacatga tagcgtggatctgaatattat  
tta tgggaagggttaattaacatactcttctacaagaccataa agtcatact  
aaa tttgggggagtgacctcgtgtacttgccagctagta agttacgtgta  
tgg tccctcaccctccctcaccctctagtcatttcgactagataa agaca  
catggttgcc ttgacgtgata tatta tttggcccaggccaaacttgatgg  
tacaactgtttgtgctcctaccactaaaa taactgatactaggtcacacatg  
gctattagggtttgttaagcttttcttaatcatccttggatgcttcgagggt 3500  
tattagggttttcaggatgtctagattgttttaaa tctcgaactctcatttc  
taggaactctggactgcacctctaggctaa tctagttta taggagcacta  
tgg tccctgaccactgatacttcactcacgatacctagggtactcgttctoga  
atgggtgtttagagttagaacagtttcacgttgacctagggtcagaacgttt  
tcattagaccgaacacgctaagatacgtgagcgacaa tcaa tgggtcaagat  
tatagtgctcctataaaactagattag tcccaagggtgcagtcattgtgca  
tagaaa tgaagagttggagatttaaacaaacctagatagtcctaaaaacctagt  
aaacctcgaaggcatccaa tga tgaactaagaaa tcttaacaaggctatta  
gcagtggtgaacgggtgtcatgagagcatttgcc tctatacttcttcgggtac  
gtcatttagctctatacaa tgaacctagggtcagttcttttagtggcgtgtcaa 4000  
gtggtaggagcacaaagagttgtaccatcaagcttggcctaggccaaa tag  
tatagcacgtcgagggaaccatgtgtctttatccagtcaaaa tgaactagg  
ggacttctaaaaaaaggctctctgcacca taaactgatacccgaagaggggtg  
agggacca tacaggtaacttagtagctgacaagcacacgagggtcgtccc  
ccaaatttagtgactttatgg tctgtggagaagagtatgttagttaacctt  
ccataaa taatgtttcacatccacgata tcatacgggtcgtattagatttta  
tcactattgtgtttgtatgcatgg ttttgcataaaaggtagatactgtagca  
gacagtttgctatttggaatggcaccgccattgttaagaagggtggacacc  
gtgtggccgaactctgata tgaacaaaa tgaagacaagacaagtgga cat  
ataaatcccatgaaccagggttggacgtaaaa caaataaa tgcctgtcg 4500  
ttttcagctgccatttcgacaaacactcatctcca ttgtccagtggggtt  
ctccttata tttcaacaaaaaatttgtttgaatgtttaaagataaaaaattg  
acttttaaaccaaaaaccgtgataacttagga tgggtgtgataa tattaag  
tcccaa ttttca ttttgaatttttaaaattgtttgaaaaaaacata tatta  
ttttattaaaaa taaagatgaagggttgacatcgaaa tcttacgagataat  
ccttatcggcgttctaacgaaattaa tactatacatgggtcttattctaaaa  
gtctatgtctttttatgtattgttttaaa tcaaaatagaattacttgaaaaat

FIG.6E

SUBSTITUTE SHEET

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gggatcttggtggttgacttgagtttgaacaaattgtcaaaatggttagct  
 gtaagtagtcgcccttctcatccatttagtttaaggatgtttcgagtt  
 taaattttcttctctcactccaagggagaatcactatgtcattatata  
 gcaaggggtgggttgattatgatagatagatgtacatttaacctgttaagt  
 aggggtcatgcggatcagagatctactttaaatggcgtagaaaaatcctgt  
 ttaaacagggtcgggaataaggattatcactctacccgtctcacttct  
 tatgtataaaatagatagaaataaaatgagaaattttatggaaatgaaat  
 tgaagtaggaggaaggcgagggacgggttaagacttccccgttctcacgc  
 tccccataaacatcacttcaactttgatgaagtagaaattttgttt  
 ggtggatatccaataagtcctcattgcactaaacaagccaggggaagagtt  
 cataaaataagacgtaaagtgtgtgtctccaacacgtaaaaacatagttact  
 cctcttgatttcccatgttaattgagaatttagagctttagtggtttaga  
 cgaggaagattgctttcactgaacactgagttgttccctaaatctatttg  
 ttacaacgaggtttatcactgcttaagtgaatgttaatttaggttttaattt  
 ctgaaaaacgtgactatgtgtaatgtcagacttgtaggactttgagcat  
 aagctctcatgtctttgatttgaactttcttaaaaagtttcataccaatg  
 gagatgtattcctcgtttataaactcaagatcattcgctaacgtgggact  
 tccttccgataatcctcaacaaaactcagtatctaacttcagtttgtttgg  
 tgaagtgttagaccctttcttttaatacactgcaatcattggtgagatttgt  
 cttttcataaaaaatattatggccttatattctatttccagcttgaaacaga  
 gttgaatgagttgtacttctctacaaatcagcccatgcacacatcagagaa  
 cccaccagacggactcatgtcttaaacaaaagggaagaaatcacatttagagg  
 gtgaagaagaaacattttacacaagtgtctcgagagcaacaatcagtcattt  
 accaaacatggatataaaaatgggtgacaaagggaagagctatcaagcaca  
 atcagggagagctcagaagaacgacaacaaataactctcaagtgaagagat  
 tttgggggtatttactacaactgcggaaaaagggtacatgtccagagatg  
 gttgggtctaagaaaaattttgttgaagcaatgttggcaacatccaanaag  
 gagaaggagaataaagggtatgcagaggcaatatgtgtcgtagaagaaga  
 cgagctagcacttatggtaataaagagagagaacatatgtattatgaggatg  
 actgaatcattgattcaggatgcttaaacacatgatttaacaatcagagt  
 ggaacaaattggatgcggagtggtccctcagagaaatgaagtagttcaaggct  
 tggaaattc

FIG. 6F

15/40

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aagcttaatgactacgaatcaa  
gctacttatcatccattaatcattttaatatacctcaatcgtctaaactca -2501  
atcatgttctcatcataattttigacgtttaactgttcgtgggttggaattg  
ggttgaattgaaacgggttcttogaatcaactaaaattgttcgagtttcaac  
tttgattaaataatgaactcaactcaacccaaccgaatcataaagttttg  
gattgaacttgtttgggtaacctaatcttatcaagcaagttcgtaatcc  
aaatgaactatataatattgagttgaagctgatcgaatttttcgaattcaa  
attttgttgattatcttctcattgttctatcaacctttgtatagttcttt  
gtcacaaaaacaaatcctcaccgaccatactattaatgttgatctaaact  
aaaaaaaaacgtttgtttatgttaacactttaatgatcataatctctagattc  
actaaaaagatcatgttacaaaacaaaatagtcgatcacaaagactatattc  
agaagccaaatttttattttaattcgactcgttttgaaatctgtgttttttt -2001  
tttttttttttttgaaatctagacgaagaataacaaaaatctctccaaaatt  
cgaatctccattgactttttggtagccgattccattaatgaacgtgggtttga  
ttttagaagccctattgaattttcttgtttgaatttattaatcttctttg  
attgcgattgaccaattgattttggttgagactcaaaaatcccaaaacatac  
aaaagtcttaattgttaacaacgaactcatgaacataatcgttaatgcataca  
tatcacaaaagcgtttcaacacatttgagtaaaagtgcgaaaagctgaa  
cttttttaaaaacaaacttcgaaccttttaactttttatatagaattgaaca  
taacaacaaaaatgttaacattgtattgacatcattatatttaacaatttt  
ccaccgaccatactactaatgttactctttaaattggaagttcttattttcg  
ttctcaaatattctaatcgtttttatcattcatcgttcaacagctactc -1500  
ttatgcattattttcttccgtttatcaattttacatttctagatccactaa  
aagttcatanaacaaaacaaaatagtcgatcccgatcgatccaccgaccat  
cttctctatagaagccaaatttttattttaattcgactcattttgaaattat  
gttatttcccaaaaattcatctccttcaactttttggtgccaatccatta  
atgaacgtgaggttggtttagaagttccattgggttttgttgatgattta  
attattttctttgccaactttttcgtaggtcaagcccatcgatttaaatat  
ttattatttgtttcttatcattttcttatcggctaaatcgatagttttct  
atgtgagcgagaaaaagcgtagtaggagattcataattgggtttgtgggatt  
gtctaaacgtgaccaattgttaggagatgcaagggaaataatgagacataca  
tgtgtgtaattcagattcagaattgtttcaaatccgagcatggatactt -1001  
cgtaaaagtgtgaanaacatgcacacctcgaacgagtgaaacaaataatt  
gcctttctttcgccccaatactcaagaaagcttgggacgctacataagaa  
gttaaattaggtatcattgaaataggatataatgttacttgatgatgta  
ttgtcatacttctcgacttcatctaatatagagtttcgaagttttcata  
ctttcccatttttgttgaaaatgtattatgcacgagtgagttggatta  
aacatctgaacccaacgagaatttaattttctcgaatttttcatttacga

FIG.7A

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tcaagcttccagaaatatttattgaaaaccttagagatcgaattaggaata  
 cagtagaagagaatgatgctcggaatgttttctagaagctcgaaaaata  
 taaaaataaatcgtagaaaaataaaaaatgtgtggtcaaagtcgaataga  
 ttttgccttccctagatatttggagaccctcgaaaaacccgagtgaaatga  
 tcatatttaggtttcggttttcctcaaaaatctaaagtgtatgaagaaatta  
 gcatatgaaaaatttagtatgtttgatcttgtcatgatttcgcacatttttc  
 ttaaaagaacctgaagtcgaatcataacggaactaggagatcgaagaaga  
 cccaagaacgggtataaacacataaaatgaagggttttgagagggggacgaa  
 agactacataagtagtatattgaggagctattattgtgtatggaggaagc  
 ccactctgagaggagatgagagactacaaaagtagatcagctgtgtctcg  
 aagcctaaaaaattgggttgtgacattgaaagttcgatttttcctaagggt  
 gacataaggggatctataacatcgtactctttgttttggttcaatttct  
 acacacacgacttgggtcggtgtttgtggcttgtctttttacatggtttc  
 aacgtgaccttgggttataaaattcactcccattttgttctttctttcgt  
 atcttaacaaccccaaaagctctcatttttagggaacacaaaaacaaacacc  
 tcaacaactttcaaATGGGGTTTCATCAAATTGACGAAAGGAACCAAGCT  
 M G F H Q I D E R N Q A  
 CTTCTCTCTAAGATCGCTATCGACGATGGCCATGGCGAGAACTCAGCCTA  
 L L S K I A I D D G H G E N S A Y  
 TTTCGATGGGTGGAAAGCTTATGATAACAATCCGTTTCACCCCGAGAATA  
 F D G W K A Y D N N P F H P E N  
 ATCCTTTGGGTGTTATTCAAATGGGTTTAGCAGAAAATCAAgtttcgtat  
 N P L G V I Q M G L A E N Q  
 atagtgttttcatgtttttcttatatcatttcacgtttgaaaaatttcgct  
 aactttgtttctgtgtgaatttcgatagCTTCTTTTGGTATGATTGTTG  
 L S F G M I V  
 ACTGGATTAGAAAACACCCCGAAGCTTCGATTTGTACACCTGAAGGACTT  
 D W I R K H P E A S I C T P E G L  
 GAGAAATTCAAAGCATTGCCAAGCTTTCAAGATTATCATGGCTTACAAGA  
 E K F K S I A N F Q D Y H G L Q E  
 GTTTCGAAAAGtactagatatgatattctaactatatctaactcagaag  
 F R K  
 cttaagtcgatggattatgatatatatatatatattttatttttcagGCG  
 A  
 ATGGCGAGTTTCATGGGGAAGGTAAGGGGTGGGAGGGTGAAATTCGACCC  
 M A S F M G K V R G G R V K F D P  
 GAGTCGGATTGTGATGGGTGGCGGTGCGACCGGAGCGAGCGAAACCGTCA  
 S R I V M G G G A T G A S E T V  
 TCTTTTGTGTTGGCGGATCCGGGGGATGCTTTTTTGGTTCCTTCTCCATAC  
 I F C L A D P G D A F L V P S P Y

-501

-1

100

500

FIG.7B

17/40

TATGCAGCgtaagtttttttttttttttttttcttttaaatctctcctttt

Y A A

cactttacata tagagagagaaaccaatttgacaaattattaactctacaa  
 attctctttgaaagtcgtatgttttgggaggggtccaaacttcaaccattc  
 taccaagtaaaccaatccacctctttcatgcctcattgctggcatacctcc  
 tcgtcttctccctatactttctttctttgtattcttctccctaaccgatg  
 tgtaatttcacaa tctactccttcgagctccagcattcttgttggcacac  
 cactttgtgtccactcccccttcgaggctcagccttctcgtagctcattg  
 ctgggtgtctggttctaata tcatttgtaacagtcraagtccaatgctag  
 tagatatgttctcgttttgggctttccctctcggacattccatcaagtt  
 tttagaacacgtctgtctaagaaaaagttttcacacccttataaataatgc  
 ttcgttctcctccctaaccgata tgggatactcactgaatattaccacatt  
 gaataaactaataacttgtgtcttctgttcttgaatagaatacaaccgg  
 atggaaagaactgatgtcaaatgataagaaaa tcaataaagggaagtaa  
 gattcggattaccttgttgaatgaatata tctcaaggcaagaacacttgttt  
 gaaattcgaatcactccacaaccaagattgatcatgtttgagcttgaatga  
 ttctgcatgcaatctaaactacatagaattacaagaacttagtcatgt  
 gctaaagaaagcacaaatgtttcttttactata tttttccaagtcggctta  
 caaatacaacatacatgacttcgtataatctcaaaatgaactat ttaag  
 gcattataagagtggttaacattcataat tttatgaccataat taaccatta  
 tgtaaatataatcttaaggtaaataaaaagccttaaaacata ttaatgaaa  
 tacaataactccaat ttttctagattgtaatccacccaaaatttataaaa  
 atgaacttcatcttcttccaatgtgacatgtggcatgaactgaatatc  
 tattttcttcccatgttcatcgaatatagtgatgattgatgtctcttg  
 gttcatatcagttctttacata tattaataacc ttttgggtacgaggtgaa  
 caatgtcgtattatgttaaaaa tactcaaaagcttttgtcctaacaatca  
 gtacgttgtttttcagGTTTGATCGAGACCTAAATGGCGAACACGAGCA

1000

1500

2000

F D R D L K W R T R A

CAAATAATTCCTGTTTCATTGCAACAGCTCGAACAACCTCCAAGTCACAGA  
 Q I I P V H C N S S N N F Q V T E  
 GGCAGCCTTAGAAATAGCCTATAAAAAGGCTCAAGAGGCCAACATGAAAG  
 A A L E I A Y K K A Q E A N M K  
 TGAAGGGTGTATAATCACCAATCCCTCAAATCCCTTAGGCACAACGTAC  
 V K G V I I T N P S N P L G T T Y  
 GACCGTGACACTCTTAAACCCTCGTCACCTTTGTGAATCAACACGACAT  
 D R D T L K T L V T F V N Q H D I

FIG.7C

SUBSTITUTE SHEET

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TCACTTAATATGCGATGAAATATACTCTGCCACTGTCTTCAAAGCCCCAA  
 H L I C D E I Y S A T V F K A P  
 CCTTCACCAGCATCGCTGAGATTGTTGAACAAATGGAGCATTGCAAGAAG  
 T F T S I A E I V E Q M E H C K K  
 GAGCTCATCCATATTCTTTATAGCTTGTCCAAAGACATGGGCCTCCCTGG  
 E L I H I L Y S L S K D M G L P G  
 TTTTCGAGTTGGAATTATTTATTCTTACAACGATGTCGTCGTCCGCCGTG  
 F R V G I I Y S Y N D V V V R R  
 CTCGGCAGATGTCGAGCTTCGGCCTCGTCTCGTCCCAGACTCAACATTG  
 A R Q M S S F G L V S S Q T Q H L  
 CTCGCCGCCATGCTTTCGACGAGGACTTTGTCGACAAATTTCTTGCCGA  
 L A A M L S D E D F V D K F L A E  
 GAACTCGAAGCGCCTGGGCGAGAGGCATGCAAGgtttgtaaactacacc  
 N S K R L G E R H A R  
 attattatttgtgggattgaaaagcattacaaaatgcaattaatttaaga  
 atgtattaatcaaatcagGTTACAAAAGAATTGGATAAAATGGGGATC  
 F T K E L D K M G I  
 ACTTGCTTGAACAGCAATGCTGGAGTTTTTGTGTGGATGGATCTACGGAG  
 T C L N S N A G V F V W M D L R R  
 GCTATTAAAAGACCAACCTTCAAAGCTGAAATGGAGCTTTGGCGTGTGA  
 L L K D Q T F K A E M E L W R V  
 TTATCAATGAAGTCAAGCTCAATGTTTCTCCTGGCTCATCCTTTCATGTC  
 I I N E V K L N V S P G S S F H V  
 ACTGAGCCAGGTTGGTTTCGAGTTTGTTCGCAAACATGGACGACAACAC  
 T E P G W F R V C F A N M D D N T  
 CGTTGACGTTGCTCTCAATAGAATCCATAGCTTTGTGCGAAAACATCGACA  
 V D V A L N R I H S F V E N I D  
 AGAAGGAAGACAATACCGTTGCAATGCCATCGAAAACGAGGCATCGAGAT  
 K K E D N T V A M P S K T R H R D  
 AATAAGTTACGATTGAGCTTCTCCTTCTCCGGGAGAAGATACGACAAGGG  
 N K L R L S F S F S G R R Y D K G  
 CAACGTTCTTAACACCGCACACGATGTCGCCTCACTCGCCATTGGTAA  
 N V L N S P H T M S P H S P L V  
 GAGCCAGAACTTATTAAagatgagtttgagaagatattatcataagtttt  
 R A R T Y \*  
 ttttagctcattaatgaatggatggatattttaaaactatgaagtgtagca  
 ctcatgctccgaaggaaatttaatttcttgattgctgaattttaagacgata  
 taaaagagaaaaaatgttttagaaaaatctaaaaaatgggagaaaaaaaag

2500

3000

FIG.7D

SUBSTITUTE SHEET



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aaaacaaattaaattttaaaaaatcagtcaaaaatcattaaagtagtacatat  
agctcatactagaagggtgagacaagactctgaaaatgatttttatgatacg  
tctttaactacgatttgcaatttcttgactgggggttactgcaatttcttgact  
gggggttacttactaagtatttctagaaaatactcaagtcacatgctactct  
tattttcaggtaaaaggcaatgtacctcttcacggacgatgacgtcgcgggt 3500  
gacgccatgagattgaagctagggtagaagtaattcagttaattttgtag  
cccttaggttagatcaaaaatcgtcttaattttattttattttatcaaa  
attttacgtgatttgttttccattttaattgtttcaataattttattatga  
acatgtaagttcatggcacattttttaaaaaatattttaaaagtttttttt  
tcgattcttaatttaatttatgcatgtagtagcgagtttatcatagtcgaag  
gaggatgttttgtagaaatgttaagctgaatgggttatgtgtaaaacggaga  
gtctactaatgtatttaagatttttatgtaaacaagcttccacttgatt  
tctgtcttgatttgctacatctcgatttcttccgtcaagaatttctctct  
aacgaaaatgataatgcacctccacatgttttatcttagcatgaacaatcg  
aattttctgttaggcaaatcgcagattgggtgttgtaagtgaagtggtatt 4000  
ggatagtcgaattttcttggtgccgactttcatcaagagtttcagtcattgt  
actttcctaactgtctcaaccgctactctgtactctacttctctagtgtg  
acaaatgatactgttgattttcttttgctacactgagaagtgttctctgaa  
ccgagcttgaacacataacctgggtgcttgatcttcgggtattgtgatattc  
tacaatgctcagcatcacgggtatctggataacttgtagcttctgcttcttt  
tatacaaacgatcataatgattgtgcctttgacatactcaagggtccgtc  
aagtcgcataccaaatgagggttctttgcaactttgtatgtactgactaatg  
actccaactccgttctcaaaatttcttcgggttggttcattgtagatctctct  
atttaactctccgtgcaagaagcattcttcatatccatctgtcgtaaatt  
tccaaatctttatttaccacaagtgtcggaggaacctatatgatgggtgatc 4500  
tttgccactgaactaaaatgtttcatcatagtcctatatgtttgagagaacc  
ctggagctacagcttgagttgtgtatcttactattcatccattcgggata  
cactttattttgtaaaatccacttgcaaaagatggatttgacatcttctgg  
tctttgtactaatcccagggttgatttatctcgaagggtataatttctt  
cctccattgtctgtgccaagccgtattgcgtgatgcttctttatcgtc  
tctagctctttactttgtcttctaaaaatagttatatgtgaacatactttgg  
atttggcttatggattctttctaaccatctaaagtcggttgagggtgcat  
ccttttcaactagggttcgcttgattgagtcactccttgctcaccaacatta  
gtgtcacttggaatttttagacacatcagcatttaagaaaaatgtgtacagt  
tttctccccgtattctgtggaagaatttcttcagtttgctcccccgctct 5000  
tctgtggaagaattc

FIG.7E

SUBSTITUTE SHEET

20/40

	10	20	30	40	
ptACCI→	tgtagttgtgtacattttttaaatcttcatcttctttaattctcttcagtt				
	tttaatttcttcacttctaaactcatttagtaaaaaaaaaATGGGATTTG				100
				M G F	
	AGATTGCAAAGACCAACTCAATCTTATCAAAATTGGCTACTAATGAAGAG				
	E I A K T N S I L S K L A T N E E				
	CATGGCGAAAACTCGCCATATTTTGATGGGTGGAAAGCATACGATAGTGA				200
	H G E N S P Y F D G W K A Y D S D				
	TCCTTTCCACCCTCTAAAAACCCCAACGGAGTTATCCAAATGGGTCTTG				
	P F H P L K N P N G V I Q M G L				
	CTGAAAATCAGCTTTGTTTAGACTTGATAGAAGATTGGATTAAGAGAAAC				300
	A E N Q L C L D L I E D W I K R N				
	CCAAAAGGTTCAATTTGTTCTGAAGGAATCAAATCATTCAAGGCCATTGC				
	P K G S I C S E G I K S F K A I A				
	CAACTTTCAAGATTATCATGGCTTGCCTGAATTCAGAAAAGCGATTGCGA				400
	N F Q D Y H G L P E F R K A I A				
	AATTTATGGAGAAAACAAGAGGAGGAAGAGTTAGATTTGATCCAGAAAGA				
	K F M E K T R G G R V R F D P E R				
	GTTGTTATGGTTGGTGGTGCCACTGGAGCTAATGAGACAATTATATTTTG				500
	V V M V G G A T G A (N) E T I I F C				
	TTTGGCTGATCCTGGCGATGCATTTTGTAGTACCTTCACCATACTACCCAG				
	L A D P G D A F L V P S P Y Y P				
	CATTTAACAGAGATTTAAGATGGAGAAGTGGAGTACAACCTTATTCCAATT				600
	A F N R D L R W R T G V Q L I P I				
	CACTGTGAGAGCTCCAATAATTTCAAATTACTTCAAAGCAGTAAAGA				
	H C E S S N N F K I T S K A V K E				
	AGCATATGAAAATGCACAAAAATCAACATCAAAGTAAAAGGTTTGATTT				700
	A Y E N A Q K S N I K V K G L I				
	TGACCAATCCATCAAATCCATTGGGCACCACTTTGGACAAAGACACACTG				
	L T N P S N P L G T T L D K D T L				
	AAAAGTGTCTTGAGTTTCACCAACCAACACAACATCCACCTTGTTTGTGA				800
	K S V L S F T N Q H N I H L V C D				
	CGAAATCTACGCAGCCACTGTCTTTGACACGCCTCAATTCGTCAGTATAG				
	E I Y A A T V F D T P Q F V S I				

FIG.8A

SUBSTITUTE SHEET

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CTGAAATCCTCGATGAACAGGAAATGACTTACTGCAACAAAGATTTAGTT 900  
A E I L D E Q E M T Y C N K D L V  
CACATCGTCTACAGTCTTTCAAAGACATGGGGTTACCAGGATTTAGAGT  
H I V Y S L S K D M G L P G F R V  
CGGAATCATATATTCTTTTAACGACGATGTCGTTAATTGTGCTAGAAAAA 1000  
G I I Y S F N D D V V N C A R K  
TGTCGAGTTTCGGTTTAGTATCTACACAAACGCAATATTTTTTAGCGGCA  
M S S F G L V S T Q T Q Y F L A A  
ATGCTATCGGACGAAAAATTCGTCGATAATTTTCTAAGAGAAAGCGCGAT 1100  
M L S D E K F V D N F L R E S A M  
GAGGTTAGGTAAAAGGCACAAACATTTTACTAATGGACTTGAAGTAGTGG  
R L G K R H K H F T N G L E V V  
GAATTAAATGCTTGAAAAATAATGCGGGGCTTTTTTGTGGATGGATTGG 1200  
G I K C L K N N A G L F C W M D L  
CGTCCACTTTTAAGGGAATCGACTTTTCGATAGCGAAATGTCGTTATGGAG  
R P L L R E S T F D S E M S L W R  
AGTTATTATAAACGATGTTAAGCTTAACGTCTCGCCTGGATCTTCGTTTG 1300  
V I I N D V K L N V S P G S S F  
AATGTCAAGAGCCAGGGTGGTTCGAGTTTGTGTTTGCAAATATGGATGAT  
E C Q E P G W F R V C F A N M D D  
GGAACGGTTGATATTGCGCTCGCGAGGATTTCGGAGGTTTCGTAGGTGTTGA 1400  
G T V D I A L A R I R R F V G V E  
GAAAAGTGGAGATAAATCGAGTTCGATGGAAAAGAAGCAACAATGGAAGA  
K S G D K S S S M E K K Q Q W K  
AGAATAATTTGAGACTTAGTTTTTCGAAAAGAATGTATGATGAAAGTGTT 1500  
K N N L R L S F S K R M Y D E S V  
TTGTCACCACTTTCGTCACCTATTCCTCCCTCACCATTAGTTCGTTAAga  
L S P L S S P I P P S P L V R \*  
cttaattaaaaggggaagaatttaattttatgtttttttatattttgaaaaa 1600  
aatttgtaagaataagattataataggaaaaagaaaataagtatgttaggat  
gaggagtattttcagaaatagttgttagcgatatgtattgacaactggtct 1700

FIG.8B

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ptACC1 → atgtacttagacatcataatttgtcttagctaattaacgaatgcaaaagt  
gaagttatgttatgactcttagaat  
ptACC2 → atgtacttagacatcataatttgtcttagctaattaatgaatgcaaaagt  
gaagttatgttatgactcttagaatcttttgatttatggactttctcga  
ptACC3 → atgtacttagacatcataatttgtcttagctaattaatgaatgcaaaagt  
gaagttatgttatg(a)<sub>19</sub>  
ptACC4 → atgtacttagacatcataatttgtcttagctaattaatgaatgcaaaagt  
gaagttatgtt(a)<sub>20</sub>  
ptACC5 → atgtacttagacatcataatttgtcttagctaattaatgaatgcaaaagt  
gaagttatgtt(a)<sub>19</sub>  
ptACC6 → atgtacttagacatcataatttgtcttagctaatt(a)<sub>58</sub>  
ptACC2 → ttatat tgtt(a)<sub>8</sub>

1800

FIG.8C

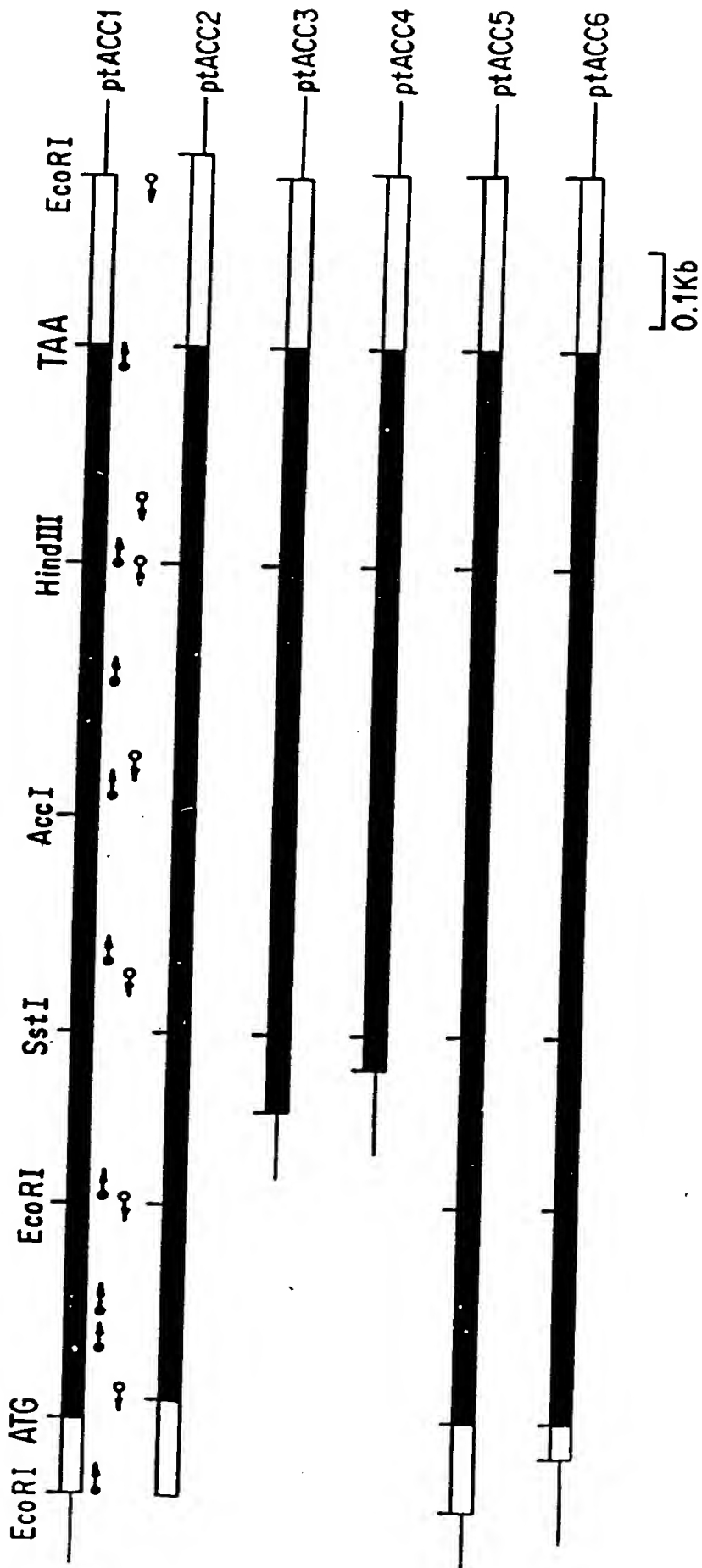


FIG. 9

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MGF HQI DERNDALLSKIALDDG HGENSPYFDGWKAYINDPFHPENNPLGV  
 MGF EIAKTN-SILSKLATNEEHGENSPYFDGWKAYISDPFHPLKNPNGV

IQMGLAENQLSFDIMVDWIRKHFEASTOTPEGLERFKSIANFQDYHGLPE  
 IQMGLAENQLCLDLIEDWIKRNPKGSIOS-EGIKSEKAIANFQDYHGLPE

CP-ACCIA

LE-ACC2

FRNAIANFMKVRGGRVKFDPSRIVMGGATGASETVIFCLADPGDAFLV  
 FRKAIKFMKTRGGRVRFDPERMVMAGGATGANETIIFCLADPGDAFLV

PSPYYAGFDRDLKWRTRAGIIRVHONGSNFQVTKAALEIAYKKAGEANM  
 PSPYYPAFNRDLRVRTGVQLPIHDESSNNFKIISKAVKEAYENACKSNI

KVKGMIIITNPSNPLGTTIYDRDTLKITLVTFVNOHDIHLICDEIYSATVFKA  
 KVKGLIITNPSNPLGTTIDKDTLKSVLSTINQHNHILVCDEIYAATVFDT

PIFTISAEIVEQ--MEHCKELIHLIYSLSKDMGLPGFRVGI IYSYNDMV  
 PGFVISAELDEQENTYCNKDLVHIYSLSKDMGLPGFRVGI IYSFNDDV

VRRARQMSSFGLVSSQTGHLAAMLSDEDFVDKFLAENSKRVGERHARFT  
 VNCARKMSSFGLVSTQTGYFLAAMLSDEKFDNFLRESAMRLGRHKHFT

KELDKMGITCLNSNAGVMVMDLRRLIKDQTFKAEMELWRVIINEVKLNV  
 NQLEVVGIKCLKNNAGFCVMDLRPLIRESTFDSEMSLWRVIINDVKLNV

SPGSSFHYTEPGVFRVCFANMDDNTVDVALNRIHSFVENIDKKEDNTVAM  
 SPGSSFECQEPGVFRVCFANMDDGTVDIALARTIRREV-GVEKSGDKSSSM

PSKTRHRDNLRLSFSFSGRRYDEGNVLNSPHTMSPH--SPLVIKKN  
 EKKQQVKKNLRLSFS--KRMDES-VL-SPLS-SPIPPSPLVR

FIG.10

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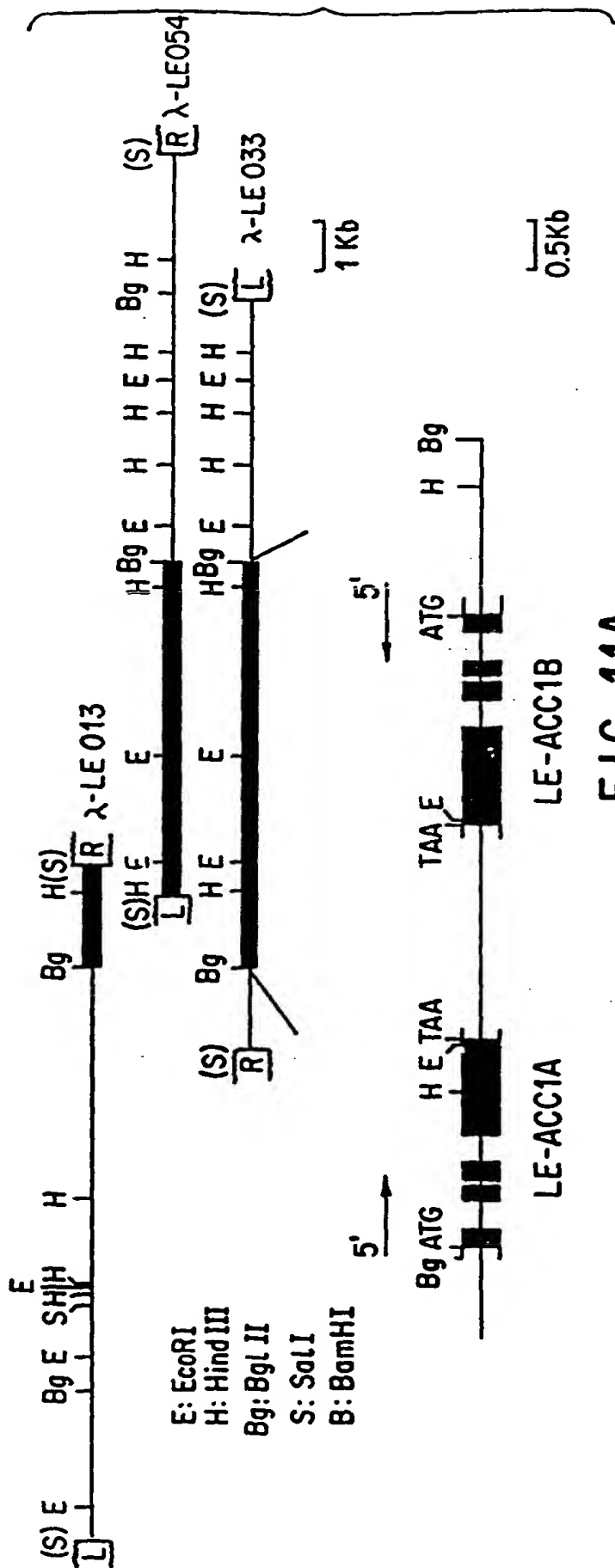


FIG. 11A

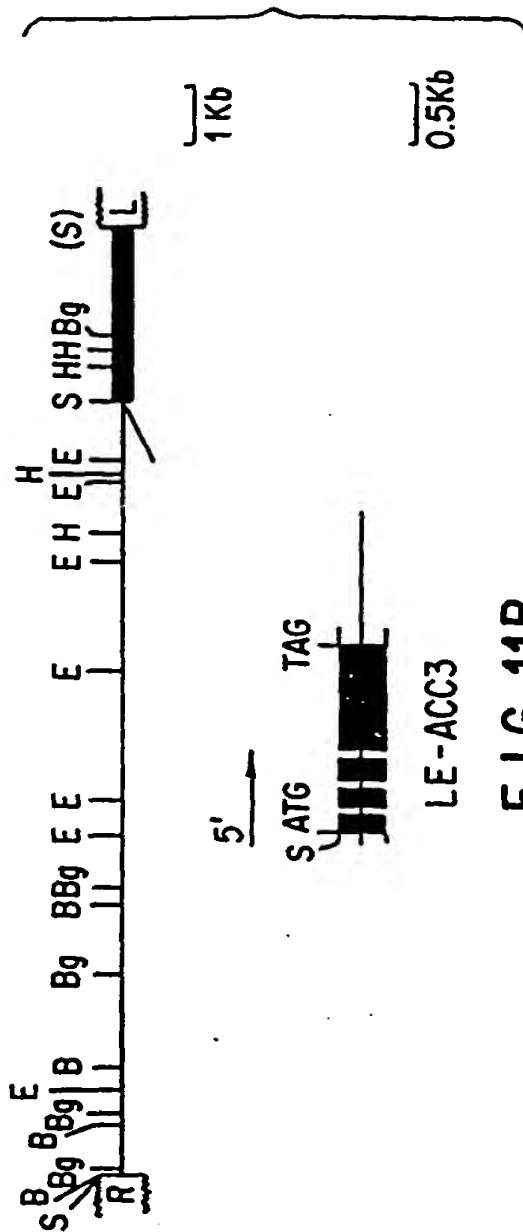


FIG. 11B





27/40

10                      20                      30                      40  
 ggatcctcattacttgtctatggctaaagtgtt  
 aaagaaattattcacaaatatctaacacatttaattgactattcaactaaatag  
 tgacgatcttttaaaaataaaaatgaagaacttaaaaattttgaccaacttcc  
 taacgatatttaattgagggatcacagatttgaatttacgcaaaaaaaaaaagaa  
 aaaaagaaatgatattactcaattataaaatttgattagagaaatagctagg  
 cctataaattgttttacattatctattcctaagttatgatattatccttca  
 atttacctgatagcgtaaaaattacaataatttgtaactaatgatgcac  
 aaaaacttaaatcattatatacacatacaaggccgagggcttaataga  
 atcgatgacctgaatcataatttctattgttttagcaatagaaaattagtta  
 tggcttcaaaatttagcgatgaattccaatgggtgtttgcattgacttaaaa  
 gatgatcaaatctactttgaagtcctgtttttgaattttgaaagtgtttga  
 taatatataaaaaataactaaaaataagttaggaagtgtttgacaaagttaa  
 atcttaaaaaataatttatcaaccaaaagttaggtctcccctattctttttt  
 ttttggacttaaaaagtcgtttaaacgtaaatttgacttataaaattttttaa  
 agttaattttaaacgggctttgtaaaagaaattaacaattcatttggaatg  
 ttaattattaaaagatccagatatgtacaaaaataaaaaataacctacctcc  
 tatagtaaaagattttcaaacaaatattaagttaaacaaagtcaaaaagttg  
 gtatatgtgaattttactagctcttgtataaaaccaatacaattagcttcgaa  
 aagtcattgatataattttctatgtgctgcttgttgggaaacttcctgta  
 acacaaagaaatgaatgaattctcccacatttttattttgtagatttaatt  
 cctattttgatatacaaaaaatattctggagaaggaaggaaatcagagcctaa  
 ccaagactaggccaaattaagcagcccatgataagcctccattcaaatgaa  
 atatacaaaatcactgtattattataagatactttgagaaatataattgtt  
 tggatcaaatagtttattacaataataattataataagtatgtgaaatga  
 tgaagctagagttttataatgaacataataatttagatttttaagttgtatat  
 tttgtctataaaatataaaattctatgaattgtaaaattatcaataatttac  
 ttaattcttttacgcaatcttactaaatataaaaagttaataactacaaa  
 agtataatcatagcatcacaaacgagctattctaaaaaaagtatcacata  
 ttttaataaatccctcccacatagtacaaacaaatcttctcatgttttgtaa  
 taataaatgatgtgaagggtttaaagggtggtgtgaataataattgcaacta  
 aaaaatttatttacatctaaaaataaaataatttaatacataaaaaatcgta  
 gatcaaaaaatttaaaatttaaatcatgatatgttaatttaatagtccagac  
 acctgcttaataaaaaactatacactatttaattgcagatgcactttataca  
 tattttgtaaaattagataaattaaatggccggctagagtaattgcaatcga  
 tagaaaagctcgatcaaaatttaacacactcaatgtgcctagtaagatct  
 tcaaatcaaaaatcaattatgattatcatctgcggtccattgttctcgctcc

-2501

-2001

-1501

FIG. 13A

SUBSTITUTE SHEET

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ctcccaggaaagtaattatccctatttatattttattttatttatataaa  
 ctacttgaaaaaggtaaaaaagaataaaataaaattaccagtagtacca -1001  
 ttgtattctcaacttttttctttctcacgtgtagcttctagcttgaacat  
 gaaatttcatataactattttagacgaaggcaattacgactaagggtatgt  
 tcgataagaaaaagaaaaatattttcttaaaaaataaaataatttttaatt  
 atttttcatatttgatttaatagcagaaaaataatttttgagggaagtatctt  
 tttttatttttgagaaaaatactttctatgaaaaataattattgatgtgaaa  
 atcaatctcgataaattgttgcaggaaacgactctgacaaatcgaaattagga  
 taaaacctcgatgaccttttaaaatcgaccttaaaatctgatccaaaactc  
 gatccagacttccgatccaaaacttgattcaagtaaaatatttttaaaaat  
 aaattctttttgacaggggtggcgtaaaaaataattttatttttaaaaatga  
 tatagtttttctaaaaataattttttgtttggttattgggggttggttcg -501  
 aggctaggggtaaaaataatttaaaacataagaaaatttttaaaagtttt  
 tgcattttttttgtgttgggggaggggcggattttgggttggaataagaaaa  
 aatattttaagataaaaaatagaatttttgaaaaataatttttcttaattttg  
 aaggaaaaatcattttttcttaaaatttgagaaaaatgaattattcttaaaaa  
 aaatttccaaaaacatttaagctaccaaaatagaaaaaaataaaaaatatt  
 ttttttctaccaaaatgcaccttaaaattagtcaaaatataccaacatttaaa  
 agagctatgaaaaaaagaaagtaagaatcgtagatcttcttttaattg  
 cgtactttttattttccaagatttgaacataaaaaatagacttttctatttt  
 tattttctgatgtaattctttatatacgtttagtcgacatgttctcattaca  
 tacttcagtcctttccctttatataatccctcacattccttaattctctt -1  
 ACACCATAACACAACACTACAACAAACACATAATACTTTTAATACAATTAGT  
 TATTTATTAGAAGTATTTAAAGTAAAGCACTTGTGAGTTGTGTACATTTT 100  
 ATTAATCTTCATCTTCTTAATTCTCTTCAGTTTTTAATTTCTTCACTTCT  
 AAACCTCATTTAGTAAAAAATAATGGGATTTGAGATTGCAAAGACCAACT  
 M G F E I A K T N  
 CAATCTTATCAAAATTGGCTACTAATGAAGAGCATGGCGAAAACCTCGCCA  
 S I L S K L A T N E E N G E N S P  
 TATTTTGATGGGTGGAAAGCATACGATAGTGATCCTTTCCACCCTCTAAA  
 Y F D G W K A Y D S D P F N P L K  
 AAACCCCAACGGAGTTATCCAAATGGGTCTTGCTGAAAATCAGgtaatta  
 N P N G V I Q M G L A E N Q  
 attatcctttatttatataattttgcagtttgaccaaacagactattataa  
 ttttttctgaaacctcgatgggtgttaaaatttctttttagCTTTGTTTA  
 L C L  
 GACTTGATAGAAGATTGGATTAAGAGAAACCCAAAAGGTTCAATTTGTTC 500  
 D L I E D W I K R N P K G S I C S

FIG.13B

SUBSTITUTE SHEET



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TGAAGGAATCAAATCATTCAAGGCCATTGCCAACTTTCAAGATTATCATG  
E G I K S F K A I A N F Q D Y N  
GCTTGCCTGAATTCAGAAAgtacatatcgtactatagtcagttaaatta  
G L P E F R K  
tattgatagtataaaaaattcgttaatatattttaactaacgagtttattta  
atcagGCGATTGCGAAATTTATGGAGAAAACAAGAGGAGGAAGAGTTAGA  
A I A K F N E K T R G G R V R  
TTTGATCCAGAAAGAGTTGTTATGGCTGGTGGTGCCACTGGAGCTAATGA  
F D P E R V V M A G G A T G A N E  
GACAATTATATTTTGTGGCTGATCCTGGCGATGCATTTTGTAGTACCTT  
T I I F C L A D P G D A F L V P  
CACCATACTACCCAGCgtaagtatatttaattatatatgtgttaaaaaaa  
S P Y Y P A  
ttaaaatcatcaaatcatttttttttatttgattaccaaaataaaattgtct  
aattttcaagattgtaacacattcatcaaagtacctaataataaaacga  
ttcagtatatttaacgatgtatataatttaattcctttggcggattgtct  
ttttatgtttgggccatcagaagaacattctggtgtatttaatttaatt  
aattaataatagatgtgttg cattcttttttaagacagcgagagtttaa  
ttagtcttaattactggattatcacgcaagctctttcttgaaattttatta  
ttctttatattaaacacatgatagcataatatctttctttttgtggaatcca  
gcttggtcgtgaagcttttgattcacacttataaaaacaacaaaaataaa  
atctggtggttaattgattaaagagagaaaataaaaaaaataatagtcaa  
tagactaataaggaaagaaaataaaaaatacacaaaatactaaaaaaaag  
aattaagggtatagtgggtctattattgagaacttttttgaaagaattgaacc  
ccactttaattttcttgcttgacccgtgaccattgcttatcgaggtaaaat  
aaaaattcaaacattgactatgacttggttagagagtaattaccacaagtc  
aaaaatttgttactctgtctcgttattttcattaggatcgataagataaca  
tctaacaatataatctttttttatttagtacttgtttatttttagtaaaagc  
acgttatacattttacaatagtcaattgttgcatatatattagtatatatat  
tttgctaagtcctaactaacaatatatttttggcaattgactaatgcagATT  
F  
TAACAGAGATTTAAGATGGAGAACTGGAGTACAACTTATTCCAATTCACT  
N R D L R W R T G V Q L I P I N  
GTGAGAGCTCCAATAATTTCAAATTAATTCAAAAGCAGTAAAGAAGCA  
C E S S N N F K I T S K A V K E A  
TATGAAAATGCACAAAAATCAAACATCAAAGTAAAGGTTTGATTTTGAC  
Y E N A Q K S N I K V K G L I L T  
CAATCCATCAAATCCATTGGGCACCACTTTGGACAAAGACACACTGAAAA  
N P S N P L G T T L D K D T L K

1000

1500

FIG. 13C

CHILDREN'S LIFE CLUB

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GTGTCTTGAGTTTCACCAACCAACACAACATCCACCTTGTTTGTGACGAA  
S V L S F T N Q N N I N L V C D E  
ATCTACGCAGCCACTGTCTTTGACACGCCTCAATTCGTCAGTATAGCTGA 2000  
I Y A A T V F D T P Q F V S I A E  
AATCCTCGATGAACAGGAAATGACTTACTGCAACAAAGATTTAGTTCACA  
I L D E Q E M T Y C N K D L V N  
TCGTCTACAGTCTTTCAAAAGACATGGGGTTACCAGGATTTAGAGTCGGA  
I V Y S L S K D N G L P G F R V G  
ATCATATATTCTTTTAACGACGATGTCGTTAATTGTGCTAGAAAAATGTC  
I I Y S F N D D V V N C A R K M S  
GAGTTTCGGTTTAGTATCTACACAAACGCAATATTTTTTAGCGGCAATGC  
S F G L V S T Q T Q Y F L A A N  
TATCGGACGAAAAATTCGTCGATAATTTTCTAAGAGAAAGCGCGATGAGG  
L S D E K F V D N F L R E S A N R  
TTAGGTAAAAGGCACAAACATTTTACTAATGGACTTGAAGTAGTGGGAAT  
L G K R N K N F T N G L E V V G I  
TAAATGCTTGAAAAATAATGCGGGGCTTTTTTGTGGATGGATTTGCGTC  
K C L K N N A G L F C W M D L R  
CACTTTTAAGGGAATCGACTTTTCGATAGCGAAATGTCGTTATGGAGAGTT  
P L L R E S T F D S E M S L W R V  
ATTATAAACGATGTTAAGCTTAACGTCTCGCCTGGATCTTCGTTTGAATG  
I I N D V K L N V S P G S S F E C  
TCAAGAGCCAGGGTGGTTCCGAGTTTGTGTTTGCAAAATATGGATGATGGAA 2500  
Q E P G W F R V C F A N N D D G  
CGGTTGATATTGCGCTCGCGAGGATTCCGAGGTTTCGTAGGTGTTGAGAAA  
T V D I A L A R I R R F V G V E K  
AGTGGAGATAAATCGAGTTCGATGGAAAAGAAGCAACAATGGAAGAAGAA  
S G D K S S S M R K K Q Q W K K N  
TAATTTGAGACTTAGTTTTTCGAAAAGAATGTATGATGAAAGTGTGTTTGT  
N L R L S F S K R M Y D E S V L  
CACCACCTTCGTCACCTATTCCCTCCCTCACCATTAGTTCGTTAAGACTTA  
S P L S S P I P P S P L V R \*  
ATTAAAAGGGAAGAATTTAATTTATGTTTTTTTATATTTTGAAAAAATT  
TGTAAGAATAAGATTATAATAGGAAAAGAAAATAAGTATGTAGGATGAGG  
AGTATTTTCAGAAATAGTTGTTAGCGTATGTATTGACAACTGGTCTATGT  
ACTTAGACATCATAATTTGTCTTAGCTAATTAATGAATGCAAAAGTGAAG  
TTATGTTATGACTCTTAGAATCTTTTGATTTATTGGACTTTCTCGATTAT  
ATTGTTAttattaaatttcatatattttatatatttataaaagtgtcgtaa 3000

FIG.13D

SUBSTITUTE SHEET

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gtcataataattgacaagataatatgaaaactttacgatcaaagataaaatt  
tgtttaaaattttaaaaatttaaagtgtgtcacataaaattgagatggagaga  
ttaatggtgtttgtgtatatattttaatggaaaaatacagtgcggtgtttgtgg  
gggattgactccagatgatagagtagaaaatggatctcctaatttttttat  
ttatgtttttactttatcgaggggtctatcaaaaaataatttatctatttttt  
aaaatagagataaaagtctgacatactctttttacttgtattttatatgtcat  
gatttttgattaggagtttggattttctctacgttcaaatacaaattaaac  
tataatgagttattttccctaataatttgagagaattatcatttggagatga  
gtacacgataataatgtcctctaatcaattacatcaaacacaaaaacatt  
attagaaattcacaaatctacatgtttgtctaatataacacatcttcatag  
ttgataagtagtacttatcatactttgtagtattatgatttcgaataactt  
gacatatgattaaattttgtaatactacattactgtttatcaaaacttggtt  
ttcgaattcatttctagtagtgtgtggcatgacttggacaagagaaaatc  
aaaatattttgaaatttatccctaataacattttatttttattttaattctat  
atataaaagaatatcgtacataatttatataataaaaattttgataatttac  
tttttatattagaaatttgataatccagtcaaaaccgccacataaaattgagcc  
aatatgtaaaatagaaaaatgttgacaaaagaaaatggatttatgggaagaca  
aactgacataggggtccaactgaaaagagttaaaattgtcggacgactttat  
aatatttttagtcaacccccacccaagccttttaaaacttagataaaatccaa  
aagataataaattttgattgataattttataatgtatcttttttatcatattg  
acatgtagaaaaattataattttataatatattttttatatagtttttaata  
tttaaaaatttttatttaaaaatattaaatgaatatattttaacttttagtta  
atcaatgactttttaaaaaacgtaatatgacaaattaaatgaatagaaaaaa  
tatcgattaaataagactttttgagatgaaaaatattgtctcatgtgaacgat  
gctaacgatgtctccaacatggattttgcttccttggctttatttcatga  
tttaatatatttatattgaaatgactaaggtaagtaaaaaaacaaaattcat  
attaaagttttgtttgagttgaattcaagttttgattatttttctattag  
aaatcagatct

3500

4000

FIG. 13E

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MGFHQIDERNQALLSKIAIDDGHGENSPYFDGWKAYDNNFFHPENNPLGV  
 MGFHQIDERNQALLSKIAIDDGHGENSPYFDGWKAYDNNFFHPENNPLGV  
 MVSISKNNQKQQLLSKIAITNDGHGENSPYFDGWKAYANNPFHLTDNPTGV  
 MVSISKNNQKQQLLSKIAITNDGHGENSPYFDGWKAYANNPFHLTDNPTGV  
 MGF-eIaktN-sILSKIAITNeeHGENSEPYFDGWKAYDSDPFHPikNPnGV  
 MkLLSekaTcnsHGaaSYFIgwgeYekNPYdeiqNPKGi

FRNAIAAnFMGKVRGGRVKFDPSRIMGGGATGASETVIFCLADPGDAFLV  
 FRKAMAsFMGKVRGGRVKFDPSRIMGGGATGASETVIFCLADPGDAFLV  
 FRQAVARFMEKVRGDRVTFDPNRIMMSGGATGAHEMLAFCLADPGDAFLV  
 FRQAVARFMEKVRGDRVTFDPNRIMMSGGATGAHEMLAFCLADPGDAFLV  
 FRKAIAKFMEKVRGGRVKFDPeRVMaGGATGANETIFCLADPGDAFLV  
 FKNAMtKFMseIRGhRVsfIsNnIMItaGATsANEJLmFLANaGDAFLI

KVKGVIITNPSNPLGTITYDRDTLKTLYTFVNQHDHILICDEIYSATVFKA  
 KVKGVIITNPSNPLGTITYDRDTLKTLYTFVNQHDHILICDEIYSATVFKA  
 KIKGLLINPSNPLGTLDDKDLRDIVTFINSKNHILVCDEIYAATVFDQ  
 KIKGLLINPSNPLGTLDDKDLRDIVTFINSKNHILVCDEIYAATVFDQ  
 KMKGIIITNPSNPLGTLDDKDLKsvLsfTnQHNHILVCDEIYAATVFDt  
 KMKGVLvTNPSNPLGTLInRneLeILLTFIdeKgIHLIsDEIYSgTVfns

VVRRARQMSSFFGLVSSDTQHLLAAMLSDEDFVDKFLAENSKRvGERHARF  
 VVRRARQMSSFFGLVSSDTQHLLAAMLSDEDFVDKFLAENSKRLGERHARF  
 VVNIARKMSSFFGLVSTDTQHLLASMLSDvFIIDKFIAESSERLGERQGMF  
 VVNIARKMSSFFGLVSTDTQHLLASMLSDEIFvEkFIAESSERLGKRQGMF  
 VVNCARKMSSFFGLVSTDTQYFLAAMLSDEKFDnFLrESamRLGKRHkhF  
 VVsaAtKMSSFFGLVSSDTQYLLscMLSDkKfttkKyIsENqKRLkKRHAMl

VSPGSSFHVTEPGWFRVCFANMDDNTVDVALNRJHSFV-----ENIDKK  
 VSPGSSFHVTEPGWFRVCFANMDDNTVDVALNRJHSFV-----ENIDKK  
 VSPGSSFHCSEPGWFRVCFANMDEITMRIALKRISYFV-----LQpKG  
 VSPGSSFHCSEPGWFRVCFANMDEITMRIALRRIRnFV-----LQtKG  
 VSPGSSFECeEPGWFRVCFANMDDgTVDIALaRIRnFV-----gveKs  
 iSPGSSCHCTEPGWFRVCFANMsdTIDIAmRRIkDFvestapnatnhqn

CONTINUED ON FIG. 14B

FIG. 14A

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IQMGLAENQLSFDIMVDWIRKHFEASICTPEGLErFKSIANFQDYHGLPE CP-ACC1A<sup>(a)</sup>  
 IQMGLAENQLSFGMIVDWIRKHFEASICTPEGLErFKSIANFQDYHGLqE CP-ACC1B<sup>(a)</sup>  
 IQMGLAENQLCFDLIQEWVNNPKASICTVEGAENFQDIAIFQDYHGLPE LE-ACC1A<sup>(b)</sup>  
 IQMGLAENQLCFDLIQEWVNNPKASICTVEGAENFQDIAIFQDYHGLPE LE-ACC1B<sup>(b)</sup>  
 IQMGLAENQLCIDLIEDWIKrNPKgSICs-EGIKsFKaIANFQDYHGLPE LE-ACC2<sup>(b)</sup>  
 IQMGLAENQLSFDLIESWlaqNPdAagfkrnGesIFreIAIFQDYHGLPa LE-ACC3<sup>(b)</sup>

PSPYYAGFDRLKVRTRAQIIrVHONrSNFQVTKAALEIAYKKAQEAM  
 PSPYYAAGFDRLKVRTRAQIIPVHONSSNFQVTEAALEIAYKKAQEAM  
 PTPYYPGFDRLRWRTGVQLFPVVCESONDFKVITKALEEAYEKAQQSNI  
 PTPYYPGFDRLRWRTGVQLFPVVCESONDFKVITKALEEAYEKAQQSNI  
 PSPYYPAFhRDLRWRTGVQLIPIHCSSNFKITsKavKEAYEnAQKSNI  
 PTPYYPGFDRLKVRTGAEIvPIHCSSNFITsALEEAYIdAKKrNI

PTFTISIAEIVEQ---MEHCKKELIHILYSLSKDMGLPGFRVGI IYSYNDV  
 PTFTISIAEIVEQ---MEHCKKELIHILYSLSKDMGLPGFRVGI IYSYNDV  
 PRFISVSEIVEd---MIECNKDLIHIVYSLSKDLGPGFRVGI VYSYNDT  
 PRFISVSEmVEE---MIECNtDLIHIVYSLSKDLGPGFRVGI VYSYNDT  
 PoFMSIAEILdEqe-MtyCNKDLVHIVYSLSKDMGLPGFRVGI IYSFNDD  
 PoFMSVreVLiEknyMktrvwErVHIVYSLSKDLGPGFRIGaIYSndem

SLSKDLGLPGFR (c)

SYAKNMGLYGERVG (d)

S<sup>TS</sup>YAKxLY (e)

TKELDKMGITOLNSNAGFFVMDLRLLKDQTFKAEMELWRVJINEMKLN  
 TKELDKMGITOLNSNAGFFVMDLRLLKDQTFKAEMELWRVJINEMKLN  
 TKGLAeVGISTLKSNAGLFFVMDLRLLKEATFDSELELWRJINEMKLN  
 TKGLAqVGISTLKSNAGLFFVMDLRLLKEATFDSELELWRJINEMKLN  
 TnGLEvVGIKQKnNAGFCVMDLRLLrEstFDSEMSLWRVJINDMKLN  
 vKGLksaGInLeSNAGFCVMDLRLLssnnFDAEMoLWkkIvyDMoLN

EDNTVAMPSKTRHRDNKLRLSFSFGRRYDEGNVLNSPHTMSPH--SPLVIAKN  
 EDNTVAMPSKTRHRDNKLRLSFSFGRRYDkGNVLNSPHTMSPH--SPLVrARTy  
 LNNIAAIAKKQCSRRKLQISLFS----RRLDh-eFmNSPAH-SPMN-SPLV--RT  
 LNNIAAIAKKQCSRSKLQISLFS----RRLDd--F-NSPAH-SPMN-SPLV--RT  
 gDksssMeKKqgwKKnLRLSFS--kRmYDEs-VL-SPIs-SPiPpSPLV--R  
 qqqsansKKkSfSKwvfrLsfndrqRer

CONTINUED FROM FIG. 14A

FIG. 14B

SUBSTITUTE SHEET

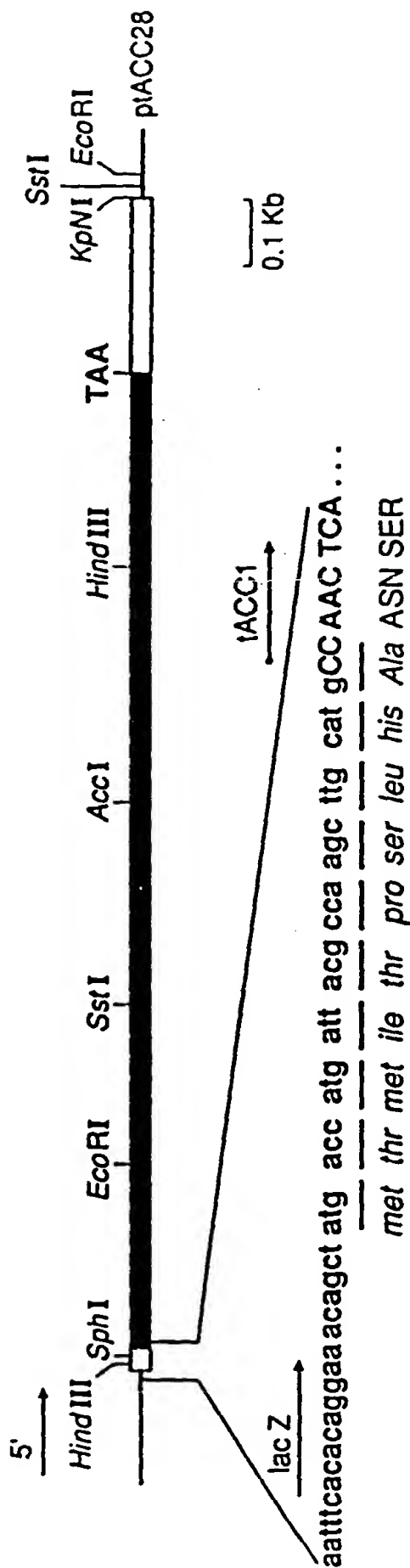


FIG. 15



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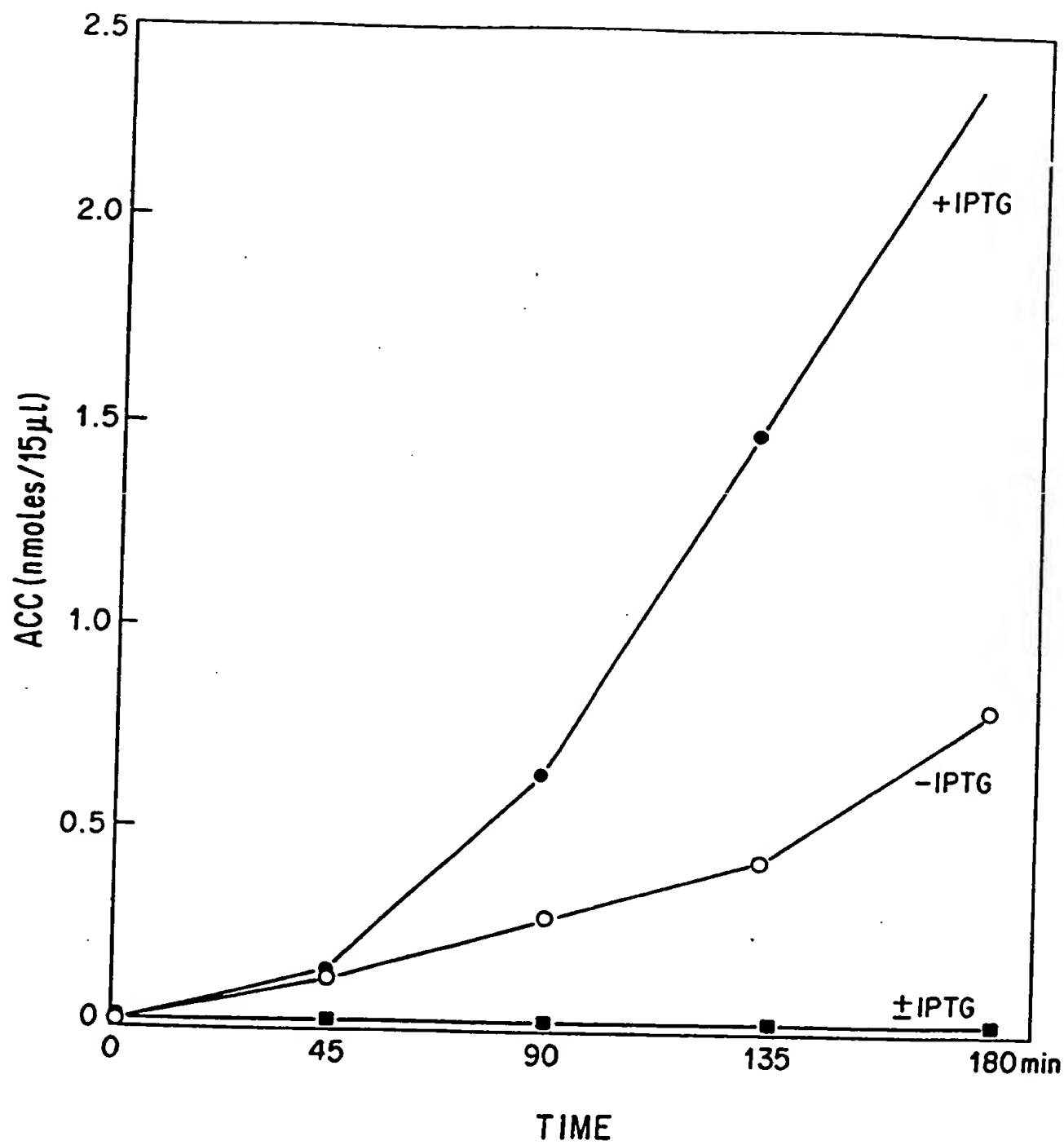


FIG. 16



FIG. 17B

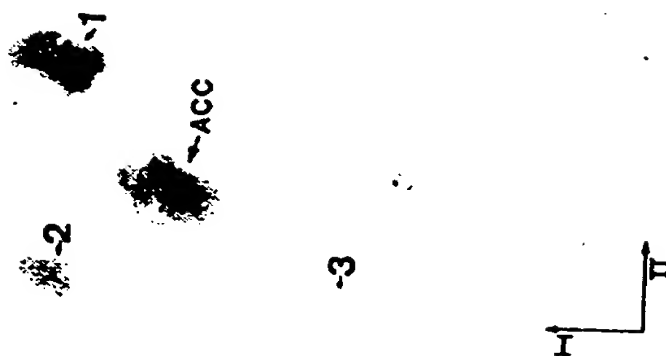


FIG. 17A

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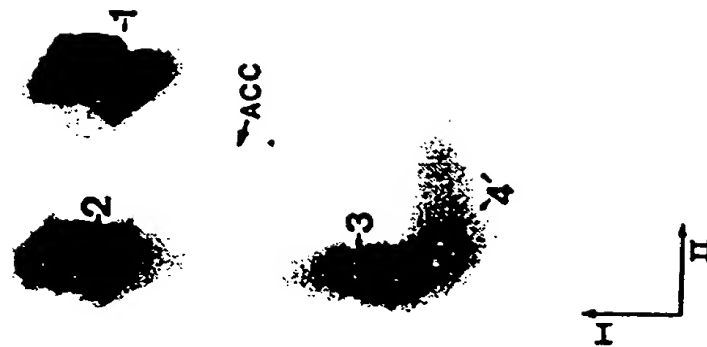
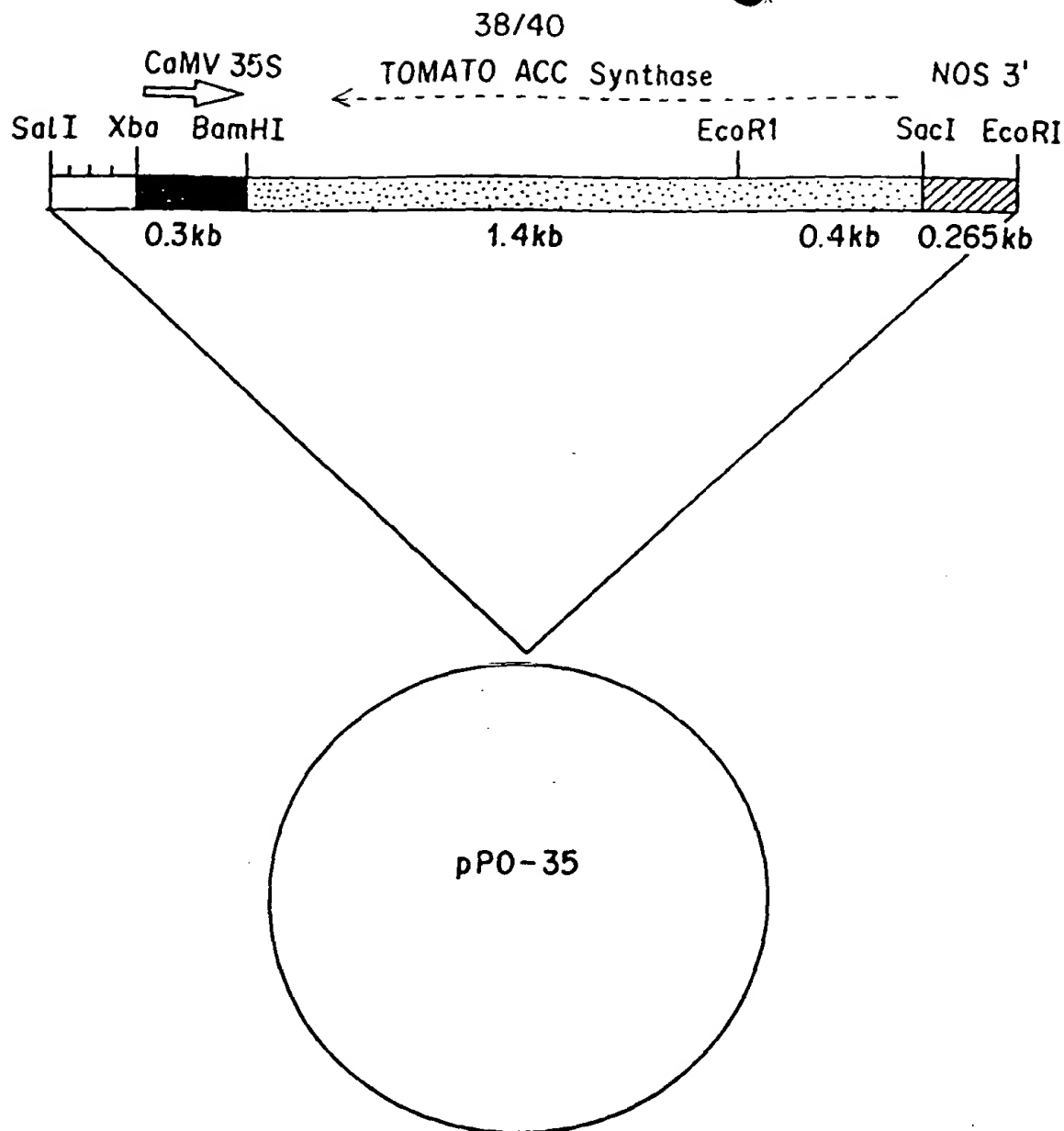


FIG. 17C



35S PROMOTER DRIVES *ANTISENSE* TOMATO ACC SYNTHASE

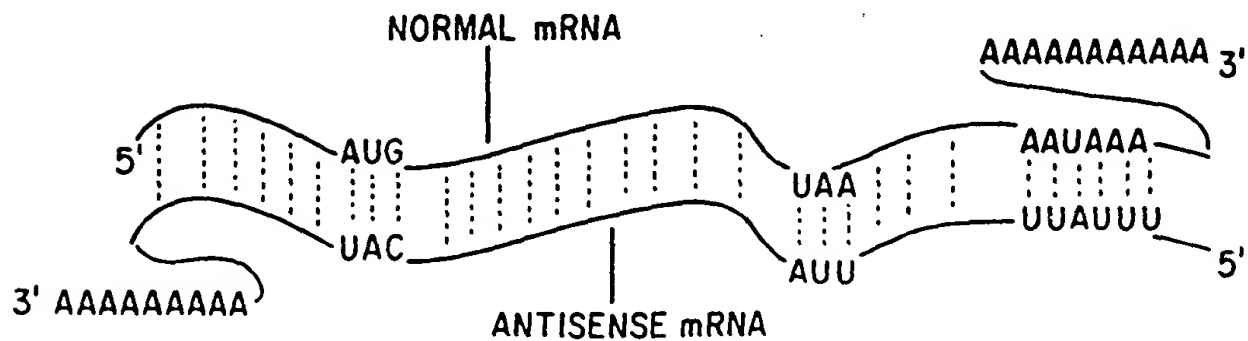


FIG. 18

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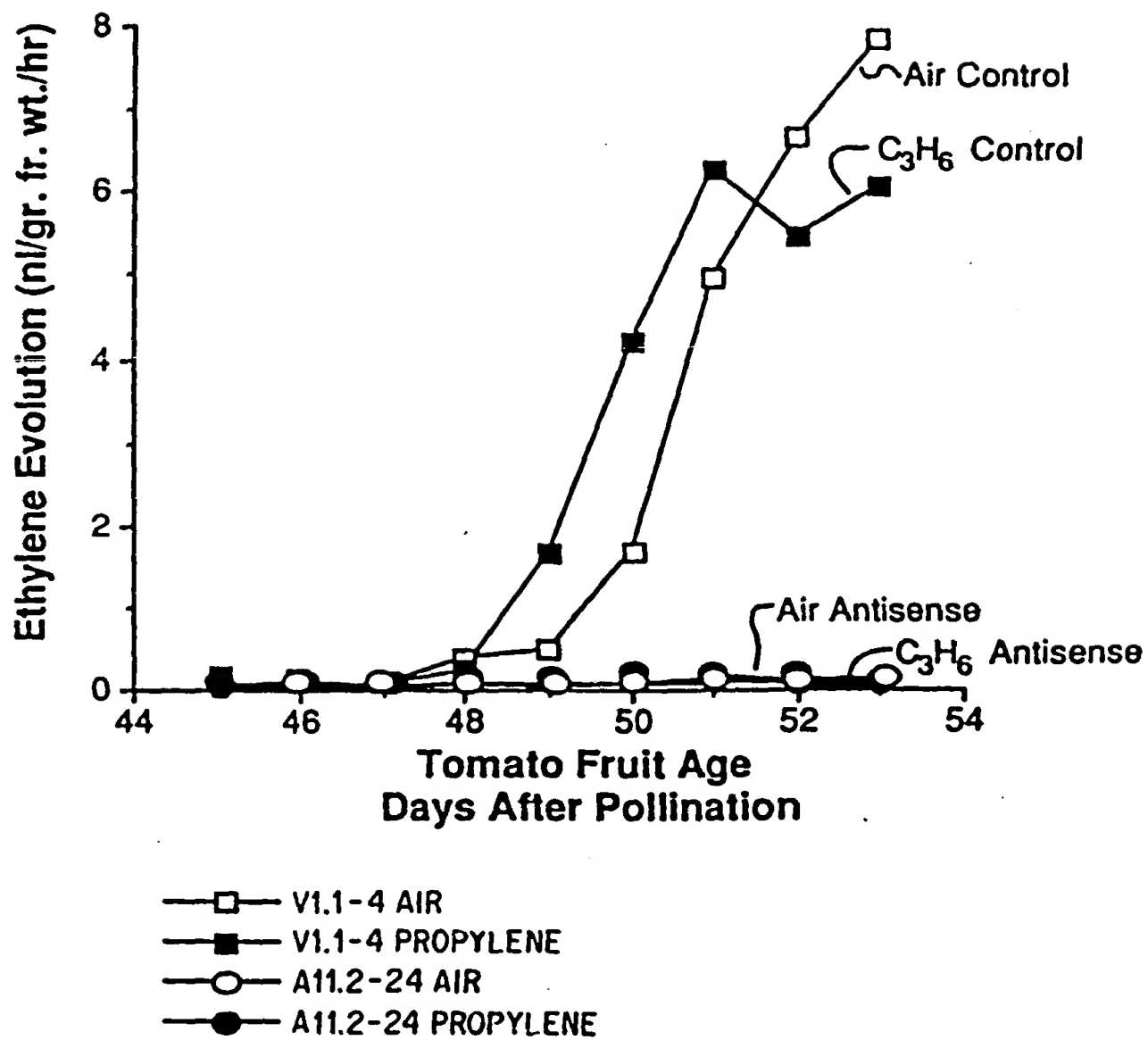


FIG. 19

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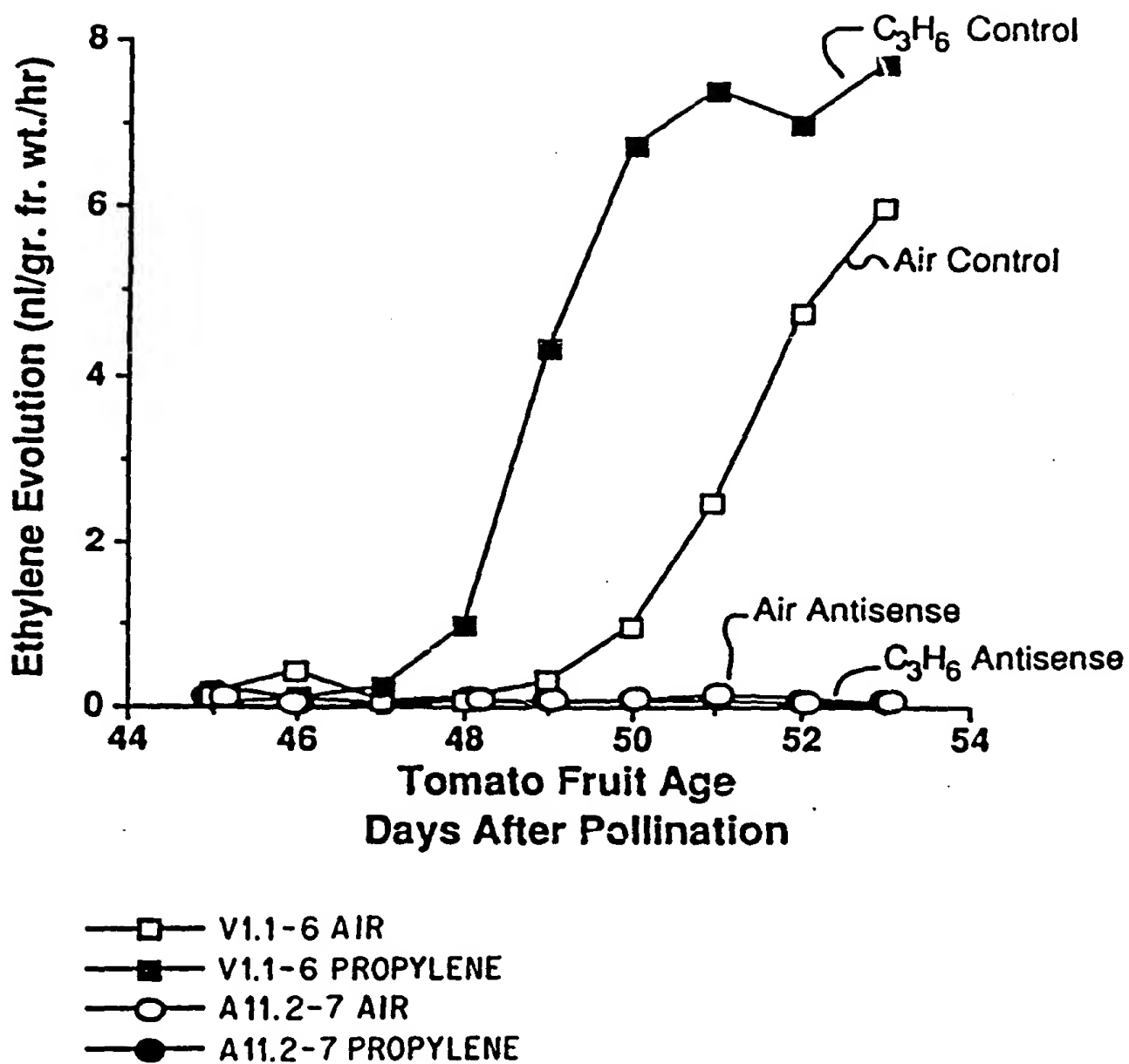


FIG. 20

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06453

## I. CLASSIFICATION OF SUBJECT MATTER (In several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC A61K 35/78

IPC(5):C12P 1/00;C12N 5/04,5/14,15/11,15/29,15/82;A01H 4/00,5/00;C12Q 1/68;C07K 13/00;C01N 33/58

US.C.:536/27;435/41,69.1,320.1,240.4,172.3,6,7,1; 800/205; 530/370,387,413

## II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
U.S.	536/27; 935/9,30,35,67, 80, 81; 530/370,387,413 435/41,161,69.1,320.1,240.4,172.3,6,7,1,240.26,70.2,70.21,182 800/205, Dig 18, Dig 44, 230

Documentation Searched other than Minimum Documentation  
to the extent that such documents are included in the fields searched \*

Chemical Abstracts Online (File Biosis &amp; Reg); USPTO Automated Patent System (File USPAT 1971-1991); Sequence Search by STIC. See attachment for search terms.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
A	Annual Review of Genetics, volume 22, Issued 1988. Weising et al.. "Foreign genes in plants: transfer, structure, expression, and applications." pages 442-443.	1-9,14, 20-21-28. 32-33, 37-38
X Y	Proceedings National Academy of Science, USA, volume 87, Issued June 1990. Van der Straeten et al.. "Cloning and sequence of two different cDNA's encoding 1-aminocyclopropane-1-carboxylate synthase in tomato," pages 4859-4863, see the entire document.	1-4,20-28 5-9,14. 16-17. 32-33, 37-38
X Y	Plant Cell Physiol., volume 31(7), Issued 1990. Nakajima, et al., "Molecular cloning and sequence of a complementary DNA encoding 1-aminocyclopropane-1-carboxylate synthase induced by tissue wounding," pages 1021-1029, see the entire document.	1,20-28 2-9,14. 16-17. 32-33, 37-38

## \* Special categories of cited documents: \*\*

-A- document defining the general state of the art which is not considered to be of particular relevance

-E- earlier document but published on or after the international filing date

-L- document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

-O- document referring to an oral disclosure, use, exhibition or other means

-P- document published prior to the international filing date but later than the priority date claimed

-T- later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

-X- document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

-Y- document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

-A- document member of the same patent family

## IV. CERTIFICATE

Date of the Actual Completion of the International Search

29 November 1991

International Searching Authority

ISA/US

Date of Mailing of the International Search Report

05 FEB 1992

Signature of Authorized Officer:

Che Swden Chereskin

## III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
<u>N</u> Y	Archives of Biochemistry and Biophysics, volume 198(1), Issued November 1979, Yu et al., "1-aminocyclopropanecarboxylate synthase, a key enzyme in ethylene biosynthesis, pages 280-286, see the entire document.	8-9 8-9, 14, 16-17, 21-28, 31-33 37-38
Y	Science, volume 244, Issued 14 April 1989, Benfey et al., "Regulated genes in transgenic plants," pages 174-181, see the entire document.	5-9, 14, 21-28, 32-33, 37-38
Y	European Journal of Biochemistry, volume 182, Issued 1989, Van der Straeten, et al., "Purification and partial characterization of 1-aminocyclopropane-1-carboxylate synthase from tomato pericarp," pages 639-647, see the entire document.	1-9, 14, 16-17, 20-28, 32-33, 37-38
Y	Methods in Enzymology, volume 152, Issued November 1987, Wallace et al., "Oligonucleotide probes for the screening of recombinant DNA libraries," pages 432-442, see the entire document.	1-9, 14, 20-28, 32-33, 37-38
Y	Proceedings National Academy Science, USA, volume 83, Issued October 1986, Bleecker et al., "Use of monoclonal antibodies in the purification and characterization of 1-aminocyclopropane-1-carboxylate synthase, an enzyme in ethylene biosynthesis," pages 7755-7759, see the entire document.	1-9, 14, 16-28, 32-33, 37-38
Y	Methods in Enzymology, volume 152, Issued November 1987, Helfman et al., "Use of antibodies to screen cDNA expression libraries prepared in plasmid vectors," pages 451-457, see the entire document.	1-9, 14, 15, 18-28, 32-33, 37-38
Y	Methods in Enzymology, volume 152, Issued November 1987, Sargent, "Isolation of differentially expressed genes," pages 423-432, see the entire document.	1-9, 14, 15, 20-28, 32- 33, 37-38
Y	Analytical Biochemistry, volume 100, Issued 1979, Lizada et al., "A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid," pages 140-145, see the entire document.	37-38



## III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication where appropriate, of the relevant passages	Relevant to Claim No
Y	US. A 4,769,061 (COMAI, et al) 06 September 1988, see the entire document.	26, 27, 32
Y	US. A 4,801,540 (HIATT, et al.) 31 January 1989, see the entire document.	23-25, 28, 33
Y	The Plant Cell, volume 1, Issued January 1989. Giovannoni et al.. "Expression of a chimeric polygalacturonase gene in transgenic <u>rin</u> (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening," pages 53-63. see the entire document.	28
Y	Biological Abstracts, volume 84(3). Issued 01 August 1987, Oaks et al.. "Molecular cloning and expression of <u>Rickettsia tsutsugamushi</u> genes for two major protein antigens in <u>Escherichia coli</u> ," page 452. Abstract 25350. see entire document.	15
Y	Biological Abstracts, volume 87(12). Issued 15 June 1989, Carniel et al.. "The gene coding for the 190,000-Dalton iron-regulated protein of yersinia species is present only in the highly pathogenic strains," pages 374-375. Abstract 125270. See entire document.	15
Y	Biological Abstracts, volume 89(2). Issued 15 January 1990, Gimeno, et al.. "Isolation and characterization of a complement DNA for carbamoyl phosphate synthetase I," page 63. Abstract 11330. see entire document.	15
P, Y	Biological Abstracts, volume 91(6). Issued 15 March 1991. Ito et al.. "Vaccination against <u>Taenia taeniaeformis</u> infection in rats using a recombinant protein and preliminary analysis of the induced antibody response." page 511. Abstract 62006, see entire document.	15

## III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P.Y	Biological Abstracts, volume 91(1), Issued 01 January 1991, Jenkinson et al., "Insertional inactivation of the gene encoding a 76-kilodalton cell surface polypeptide in <u>Streptococcus gordonii</u> Challis has a pleiotropic effect on cell surface composition and properties." pages 407-408, Abstract 3878, see entire document.	15
P.Y	The Journal of Biological Chemistry, volume 266(6), Issued 25 February 1991, Sato, et al.. "The 1-aminocyclopropane- 1-carboxylate synthase of <u>Cucurbita</u> ," pages 3752-3759, see the entire document.	1-9.14-28. 32-33. 37-38

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter<sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>13</sup>, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>1</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See attachment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-9, 14-28, 32, 33, 37-38

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

## Attachment to PCT/ISA/210

## Search terms

acc synthase - equivalents

cdna

genomic

sequence?

tomato

zucchini

lycopersicum

cucurbita

antibod?

librar?

screen?

induc?

anti sense

antisense

mutat?

lambdagt11

gt11

affinity purif?

subtract?

uninduc?

## Attachment

### Reasons for Holding Lack of Unity of Invention

Group I, claims 1-9, 14, 20, and 37-38, drawn to purified DNA encoding ACC synthase, expression vectors, probes, recombinant host cells where the host cell is a plant, method of using plant host cells to prepare ACC synthase, and method of use to produce ethylene using plant host cells.

Group II, claims 1-9, 14, 20, and 37-38, drawn to purified DNA encoding ACC synthase, expression vectors, probes, recombinant host cells where the host cell is a bacteria, method of using bacterial host cells to prepare ACC synthase, and method of use to produce ethylene using bacterial host cells.

Group III, claims 1-9, 14, 20, and 37-38, drawn to purified DNA encoding ACC synthase, expression vectors, probes, recombinant host cells where the host cell is a yeast, method of using yeast host cells to prepare ACC synthase, and method of use to produce ethylene using yeast host cells.

Group IV, claims 1-9, 14, 20, and 37-38, drawn to purified DNA encoding ACC synthase, expression vectors, probes, recombinant host cells where the host cell is an alga cell, method of using algal host cells to prepare ACC synthase, and method of use to produce ethylene using algal host cells.

Group V, claims 10-13, drawn to plant material.

Group VI, claim 15, drawn to a method to obtain inducible DNA sequences.

Group VII, claims 16-17, drawn to ACC synthase enzyme.

Group VIII, claims 18-19, drawn to antibody compositions.

Group IX, claims 26, 27, and 32, drawn to a nucleotide sequence encoding a mutated ACC synthase, expression system and transformed plant.

Group X, claims 21-25, 28, and 33 drawn to antisense nucleotide sequences, expression systems, plants with insufficient ACC synthase and method of making such plants.

Group XI, claims 29 and 34, drawn to ripening resistant plants and method of making.

Group XII, claims 30 and 35, drawn to senescence resistant plants and method of making.

Group XIII, claims 31 and 36, drawn to plants which do not develop in a normal time frame and method of making.

Group XIV, claim 39, drawn to a method of using ethylene in fruit ripening.

Group XV, claim 40, drawn to a method of using ethylene to make ethanol.

Groups I-IV are independent inventions because each group is drawn to a different class of organism: plant, bacteria, yeast, or algae. Thus, the expression vectors, recombinant host cells and corresponding methods of use will be different for each of the four groups. For example, the expression of any given sequence in a particular cell type requires different control sequences. Thus, a plant expression system is not interchangeable with a bacterial expression system, for example. Furthermore, even though expression systems for all four species are known, expression in any given cell type is unpredictable. For example, a bacterial structural gene may be expressed well in a bacterial cell with the proper control sequences, and may not be expressed at all in a plant cell with the appropriate control signals.

Groups V and IX-XIII are unrelated to Groups II-IV because groups II-IV are not drawn to plants.

Group I and Group V are related as mutually exclusive species in intermediate-final product relationship. Distinctness is proven for claims in this relationship if the intermediate product is useful to make other than the final product and the species are distinct.

In the instant case, the intermediate product is deemed to be useful as a means to produce a product in culture such as ACC synthase or ethylene and the inventions are deemed distinct since there is nothing on this record to show them to be obvious variants. Should applicant traverse on the ground that the species are not distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case.

Inventions VI and I-IV are related as process of making and product made. The inventions are distinct if either or both of the following can be shown: (1) that the process as claimed can be used to make other and materially different product or (2) that the product as claimed can be made by another and materially different process. In the instant case the process as claimed can be used to make other and materially different product. The method of Group VI is not restricted to ACC synthases and can be used with any inducible sequence.

The DNA, vectors and probes of Groups I-IV and Group VII are related as mutually exclusive species in intermediate-final product relationship. Distinctness is proven for claims in this relationship if the intermediate product is useful to make other than the final product, and the species are distinct.

In the instant case, the intermediate product is deemed to be useful as a hybridization probe and the inventions are deemed distinct since there is

nothing on this record to show them to be obvious variants. Should applicant traverse on the ground that the species are not distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case.

Groups I-IV and Group VIII are independent and wholly separate products. The products are unrelated structurally. Likewise, methods to obtain the products of Group I-IV would involve totally different process steps from methods to obtain the products of Group VIII. Likewise, the methods of use of Groups I-IV could not be used with the products of Group VIII.

Each of Groups I, and IX-XIII are independent and wholly separate products. The products of each group would have structural differences and different properties, and methods of making and using the products recited in each of these groups would require different method steps.

Groups I-IV and either of Groups XIV and XV are independent and distinct inventions. The method of making ethylene of Groups I-IV can be practiced separately and independently of the methods of using ethylene of Groups XIV and XV and would recite different process steps.

Group V and Group VI are independent and distinct inventions. The method of Group VI cannot be used to make the product of Group V and the product of Group V would not be produced by the method of Group VI.

Groups V and VII are related as mutually exclusive species in intermediate-final product relationship since the plant material of Group V could be used as a source of the ACC synthase of Group VII. Distinctness is proven for claims in this relationship if the intermediate product is useful to make other than the final product, and the species are distinct.

In the instant case, the intermediate product is deemed to be useful as



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a source of plant produce such as fruits, vegetables or nuts and the inventions are deemed distinct since there is nothing on this record to show them to be obvious variants. Should applicant traverse on the ground that the species are not distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case.

Groups V and VIII contain independent and wholly separate products. The two products are unrelated structurally. Likewise, methods to obtain and methods to use the two products would involve totally different process steps.

Each of Groups V, and IX-XIII are independent and wholly separate products. The products of each group would have structural differences and different properties, and methods of making and using the products recited in each of these groups would require different method steps.

Groups V and either or Groups XIV or XV are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. In the instant case the product as claimed can be used in a materially different process of using that product such as to produce fruit, vegetables or nuts.

The method of Group VI is independent and distinct from the products of Groups VII-VIII, and XI-XIII because the products of Groups VII-VIII, and XI-XIII could not be used in the process of Group VI and would not be the product of the method of Group VI.

Group VI and either of groups IX and X are related as process of making and product made. The inventions are distinct if either or both of

the following can be shown: (1) that the process as claimed can be used to make other and materially different product or (2) that the product as claimed can be made by another and materially different process. In the instant case the process as claimed can be used to make other and materially different product. The use of the method of Group VI is not limited to ACC synthase sequences but can be used with any inducible sequence.

Groups VI and either of Groups XIV or XV are independent and distinct inventions. The method of Group VI can be practiced separately and independently of the method of either of Groups XIV or XV and recites different process steps.

Group VII and Group VIII are related as mutually exclusive species in intermediate-final product relationship. Distinctness is proven for claims in this relationship if the intermediate product is useful to make other than the final product, and the species are distinct.

In the instant case, the intermediate product is deemed to be useful to produce ethylene and the inventions are deemed distinct since there is nothing on this record to show them to be obvious variants. Should applicant traverse on the ground that the species are not distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case.

Any of Groups IX through XIII and Group VII are related as mutually exclusive species in intermediate-final product relationship since the plants of Groups IX-XIII could be used as the source of ACC synthase of Group VII. Distinctness is proven for claims in this relationship if the intermediate product is useful to make other than the final product, and the species are distinct.

In the instant case, the intermediate product is deemed to be useful as

a source of fruits, nuts or vegetables and the inventions are deemed distinct since there is nothing on this record to show them to be obvious variants. Should applicant traverse on the ground that the species are not distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case.

Groups VII and either of Groups XIV-XV are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. In the instant case the product as claimed can be used in a materially different process of using that product such as to make antibodies to ACC synthase.

Group VIII and any of Groups IX-XIII are independent and wholly separate products. The products are unrelated structurally. Likewise, methods to obtain the products would involve totally different process steps.

Group VIII and either of Groups XIV-XV are independent and distinct inventions. The product of Group VIII cannot be used in the method of Groups XIV-XV and would not be the product produced by the methods of Groups XIV-XV.

Any of Groups IX-XIII and either of Groups XIV-XV are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. In the instant case the product as claimed can be used in a materially different process of using that product such as to produce fruits, vegetables or nuts.

Groups XIV and XV are independent and distinct inventions. The method of Group XIV can be practiced separately and independently of the method of Group XV and recites different process steps.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter, fall into different statutory classes of invention and are separately classified and searched, restriction for examination purposes as indicated is proper.